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(71) Applicant: RESEARCH FOUNDATION OF CUNY,  
HUNTER COLLEGE [US/US]; 79 Fifth Avenue, New  
York, NY 10003 (US).(72) Inventor: FILBIN, Marie, T.; Apartment 3D, 215 West 78  
Street, New York, NY 10024 (US).(74) Agents: HALEY, James, F., Jr. et al.; Fish & Neave, 1251  
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THEREOF

## (57) Abstract

This invention relates to the novel identification of myelin-associated glycoprotein ("MAG") as a potent inhibitor of neural regeneration. More particularly, this invention relates to compositions and methods useful for reversing inhibition of neural regeneration in the central and peripheral nervous system. Assays to monitor the effects of MAG on neural regeneration and to identify agents which will block or promote the inhibitory effects of MAG on neural outgrowth are provided. Screening methods for identifying such agents are also provided. This invention also relates to compositions and methods using agents that can reverse the inhibitory effects of MAG on neural regeneration. Methods for regulating and for promoting neural growth or regeneration in the nervous system, methods for treating injuries or damage to nervous tissue or neurons, and methods for treating neural degeneration associated with disorders or diseases, comprising the step of administering at least one of the compositions according to this invention are provided.

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COMPOSITIONS AND METHODS USING MYELIN-ASSOCIATED  
GLYCOPROTEIN (MAG) AND INHIBITORS THEREOF

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TECHNICAL FIELD OF THE INVENTION

This invention relates to the novel identification of myelin-associated glycoprotein ("MAG") as a potent inhibitor of neural regeneration. More particularly, this invention relates to compositions and methods useful for reversing inhibition of neural regeneration in the central and peripheral nervous system. Assays to monitor the effects of MAG on neural regeneration and to identify agents which will block or promote the inhibitory effects of MAG on neural outgrowth are provided. Screening methods for identifying such agents are also provided. This invention also relates to compositions and methods using agents that can reverse the inhibitory effects of MAG on neural regeneration. Methods for regulating and for promoting neural growth or regeneration in the nervous system, methods for treating injuries or damage to nervous tissue or neurons, and methods for treating neural degeneration associated with disorders or diseases, comprising the step of administering at least one of the compositions according to this invention are provided.

### BACKGROUND OF THE INVENTION

The mammalian nervous system does not regenerate after injury despite the fact that there are many molecules present which encourage/promote axonal (nerve) growth. It is believed that the lack of regeneration caused by the presence of molecules in the central nervous system (CNS) and the peripheral nervous system (PNS) which actively prevent/inhibit regeneration. Hence, the well documented inability of the adult mammalian CNS to regenerate after injury is believed to result from a predominance of inhibitory molecules.

It has been demonstrated that when neurons are grown on tissue sections of the CNS they fail to extend processes onto areas of white matter, myelin. It is believed that myelin-specific inhibitory molecules can largely account for the lack of CNS regeneration and their identification will help in the design of therapies to encourage regrowth after injury. The precise molecules responsible for this inhibition have, so far, remained elusive. If these inhibitory molecules can be identified and blocked, then neural regeneration can be encouraged.

Schwab and co-workers have identified two components in CNS myelin, in the molecular weight ranges of approximately 35kD and 250kD, which arrest axonal growth. The most compelling observation in support of the inhibitory action of these two protein fractions is that antibodies raised to proteins eluted from these regions of polyacrylamide gels after separation of CNS myelin proteins, specifically reverses the inhibitory effect of myelin in vitro and allows limited spinal cord regeneration when applied in vivo to transected nerves (Caroni, P. and Schwab, M. E., Neuron, 1, pp. 85-96 (1988a); J. Cell Biol., 106, pp. 1281-88 (1988b); Schnell, L. and Schwab, M. E., Nature, 343, pp. 269-72 (1990)). The nature of these two proteins and how they act have not yet been described, but, it is generally accepted that they are significant contributors to the inhibitory effect of this tissue.

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However, as acknowledged by the authors, other factors are likely to contribute to the inhibition by CNS myelin as even in the presence of antibodies directed against these two proteins, the majority of axons in vivo fail to  
5 regenerate (Schnell, L. and Schwab, M. E., Nature, 343, pp. 269-72 (1990); Schnell et al., Nature, 367, pp. 170-73 (1993)).

In addition to inhibitory molecules in myelin, another family of proteins has recently been identified  
10 whose members inhibit axonal regeneration. These molecules are called collapsins (Luo et al., Cell, 75, pp. 217-27 (1993)). However, collapsins are found ubiquitously throughout the nervous system and as they are found in  
15 i.e. gray matter, they are unlikely to contribute significantly to the lack of neural regeneration after injury. Instead, the collapsins most likely play a role in guiding growing axons during development.

Previously it was shown that MAG, like many  
20 members of the Ig-superfamily of molecules, could promote neurite outgrowth, in this case, from dorsal root ganglion (DRG) neurons from 2 day old rats (13). We observed a similar effect on DRG neurons from rats up to postnatal day 3, but after this age MAG had the opposite effect, i.e., it  
25 inhibited neurite outgrowth (Mukhopadhyay et al., Neuron, 13, pp. 757-67 (1994)). Furthermore, we also found that MAG dramatically inhibited neurite outgrowth from cerebellar neurons from rats of all ages up to adult. Polyclonal antibodies directed against MAG could  
30 specifically block both stimulatory and inhibitory effects of MAG on neurite outgrowth. MAG, therefore, depending on the age and the type of neuron, can either promote or inhibit neurite outgrowth. Subsequent to our report on the inhibitory effects of MAG, another group demonstrated,  
35 using a different complementary approach, that MAG is an inhibitor of axonal growth (McKerracher et al., Neuron, 13,

pp. 805-811 (1994); WO 95/22344 (24 August 1995);  
incorporated herein by reference).

It would be useful to block the inhibitors of axonal regeneration for treating patients with nervous system injuries where neural regeneration is a problem. No molecule had been identified in myelin which is a potent inhibitor of axonal regeneration. Although Schwab and co-workers identified components in myelin that are inhibitory, the precise nature of these components has not been identified, i.e., they have not been cloned nor have the proteins been purified. In addition, there was no information available on the component on the neuron that the putative inhibitory molecules interact with to prevent regrowth. As no inhibitory nor interacting molecules had been precisely identified, it was difficult, if not impossible, to logically design strategies whereby these molecules can be blocked and prevented from inhibiting neural regeneration.

#### SUMMARY OF THE INVENTION

The present invention solves the problems referred to above by identifying MAG as a potent inhibitor of axonal regeneration in the central nervous system (CNS) and the peripheral nervous system (PNS). The present invention provides compositions and methods for blocking or manipulating the levels of MAG activity in the nervous system.

In one embodiment, the compositions comprise a pharmaceutically acceptable carrier and a therapeutically effective amount of at least one inhibitor of MAG. Inhibitors of MAG include but are not limited to anti-MAG antibodies, altered and/or mutated forms of MAG characterized by an altered biological activity, free sialic acid-bearing sugars, modified derivatives of sialic acid attached to a sugar, a sialic acid-bearing sugar attached to a protein or lipid carrier molecule, a modified

sialic acid-bearing sugar attached to a protein or lipid carrier molecule and a sialic acid glycopeptide.

In one preferred embodiment, the MAG inhibitor comprises a small sialic acid-bearing oligosaccharide  
5 (sugar), which is optionally a competitive inhibitor of sialidase. More preferably, the sialic acid analog is sialo 2,3- $\alpha$  lactose (2,3-SL) or 2,3-dideoxy sialic acid (DD-NANA).

In another preferred embodiment, the MAG  
10 inhibitor comprises an altered and/or mutant form of MAG which can inhibit the binding of endogenous MAG to neurons in the CNS or PNS. Altered forms of MAG preferably comprise all or a portion of the extracellular domain of  
15 MAG fused to another molecule which renders the chimeric protein soluble. One such preferred soluble MAG chimeric protein comprises the five Ig-like domains of MAG fused to the Fc domain of a human immunoglobulin molecule, such as IgG ("MAG-Fc").

Preferred altered/mutated forms of MAG are  
20 soluble molecules which harbor one or more mutations in the MAG molecule that reduce or eliminate its ability to inhibit or promote neurite outgrowth compared to endogenous MAG or MAG-Fc, but do not significantly diminish the  
binding of the altered or mutant form of MAG to neuronal  
25 surfaces. Most preferred altered/mutant forms of MAG are soluble molecules comprising a truncated form of MAG-Fc consisting of the first three of the five extracellular Ig-like domains of MAG fused to an immunoglobulin Fc domain ("MAG(d1-3)-Fc").

30 In another embodiment, the compositions comprise a therapeutically effective amount of an enzyme that can alter or remove sialic acid residues having a Neu5Ac $\alpha$ 2-3Gal $\beta$ 1-3GalNAc (3-O) structure, which mediate MAG binding to neuronal surfaces in the PNS or CNS. Preferred  
35 compositions of this embodiment comprise sialidase (a neuraminidase) and sialyl transferases that alter the

structure and/or lower the effective concentration of Neu5Ac $\alpha$ 2-3GalB1-3GalNAc ("3-O") sialylated glycans.

The present invention also provides methods for regulating and for promoting neural growth or regeneration in the nervous system, methods for treating injuries or damage to nervous tissue or neurons, and methods for treating neural degeneration associated with disorders or diseases, comprising the step of administering at least one of the pharmaceutical compositions according to this invention.

The present invention provides an assay for determining whether neurite outgrowth from a particular type of neuron at a particular age is stimulated or inhibited in the presence of MAG (or a MAG derivative). In one embodiment, the method comprises the steps of:

- a) culturing a first sample of a selected neuronal cell type on a growth-permissive substrate in the absence of MAG;
- b) culturing a second sample of the selected neuronal cell type on a growth-permissive substrate comprising bound MAG; and
- c) comparing the relative amount of neurite growth in the cultured cells of a) and b); wherein when the relative growth of neurites in the cultured cells of a) is greater than in b), the neuronal cell is inhibited by the presence of MAG, and when the relative growth of neurites in the cultured cells of a) is less than in b), the neuronal cell is stimulated by the presence of MAG.

In a preferred embodiment, the growth-permissive substrate in the absence of MAG comprises a monolayer of mammalian cells that do not express cell-surface MAG, and the growth-permissive substrate comprising bound MAG comprises a monolayer equivalent mammalian cells engineered to express cell surface MAG. Preferably, the mammalian cells are CHO cells engineered to express cell surface MAG, such as CHO-MAG2 cells.



The present invention also provides methods for identifying a MAG-dependent neurite growth altering agent, i.e., an agent which alters neurite outgrowth from a selected neuronal cell type, or population of mixed cell types, in the presence of MAG compared to the absence of MAG.

In one embodiment, the method comprises the steps of:

- a) culturing a first sample of a selected neuronal cell type on a growth-permissive substrate in the absence of MAG;
- b) culturing a second sample of the selected neuronal cell type on a growth-permissive substrate comprising bound MAG in an amount sufficient to alter neurite outgrowth from the cells compared to the first sample of cells cultured in the absence of MAG;
- c) incubating the cell cultures of a) and b) with a known relative concentration of a test agent for a time sufficient to allow neurite growth; and
- d) comparing the relative amount of neurite growth in the cultured cells of a) and b); wherein an agent that changes the relative growth of neurites in the cultured cells of a) and b) is identified as a MAG-dependent neurite growth altering agent.

In a preferred embodiment, the growth-permissive substrate in the absence of MAG comprises a monolayer of mammalian cells that do not express cell-surface MAG, and the growth-permissive substrate comprising bound MAG comprises a monolayer equivalent mammalian cells engineered to express cell surface MAG. Preferably, the mammalian cells are CHO cells engineered to express cell surface MAG, such as CHO-MAG2 cells.

In another embodiment of this invention, the method for identifying a MAG-dependent neurite growth altering agent comprises the steps of:

- a) culturing separate samples of a selected neuronal cell type on a growth-permissive substrate lacking MAG;

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- b) culturing a first sample of a) with a known concentration of a traceable, soluble form of MAG;
- c) culturing a second sample of a) with a known concentration of a traceable, soluble form of a control protein lacking MAG activity;
- d) incubating the cultures of b) and c) with a known relative concentration of a test agent for a time sufficient to allow neurite growth; and e) comparing the relative amount of neurite growth in the cultured cells of c) and d); wherein an agent that changes the relative growth of neurites in the cultured cells of c) and d) is identified as a MAG-dependent neurite growth altering agent.

In one preferred embodiment, the growth-permissive substrate lacking MAG comprises a monolayer of mammalian cells that do not express cell-surface MAG, such as COS or NIH 3T3 cells. In another preferred embodiment, the growth-permissive substrate lacking MAG comprises an immobilized monolayer of a purified, growth-promoting factor. One most preferred neuronal growth-promoting factor which may be immobilized onto a monolayer is the L1 glycoprotein.

In preferred embodiments, the soluble form of MAG is a MAG-Fc fusion protein, and the soluble control protein lacking MAG activity is a MUC 18-Fc fusion protein. Preferred traceable fusion proteins are radioactively or fluorescently labeled.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1. Inhibition of Neurite Outgrowth from Cerebellar Neurons by MAG. Cerebellar neurons from post-natal day (PND) 1, 4 and 7 were grown overnight on a monolayer of MAG-expressing (clear bars) or control transfected CHO cells (hatched bars). Neurons were stained for GAP-43

antigen and neurite length was measured and the average length calculated from at least 150 measurements (+/- SEM).

**Fig. 2. Effect of MAG on Neurite Outgrowth from DRG Neurons of Different Ages.** DRG neurons were isolated from animals from PND 1 to PND 20 and grown on MAG-expressing or control CHO cells and neurite length was calculated as described in Fig. 1.

**Fig. 3. Effect of MAG on Neurite Outgrowth from Different Neuronal Cell Types.** Various neurons from PND1 animals were isolated and grown overnight on MAG-expressing (dark hatched) or control (light hatched) CHO cells. Average neurite length was calculated as described in Fig. 1. RG = retinal ganglion; HN= hippocampal; MN = motor; and SCG = superior cervical ganglion neurons.

**Fig. 4. Sialic acid-dependent Binding of MAG to Cerebellar and DRG Neurons.** Radiolabelled MAG-Fc (solid bars) was allowed to bind to PND 1 cerebellar and DRG neurons. Incubations were also carried out in the presence of 5  $\mu$ g/ml of MAG 513 monoclonal antibody (dark hatched bars) or with neurons that had been desialyated (speckled bars). Each experiment was carried out in quadruplicate. The results are the mean of 3 experiments.

**Fig. 5. Desialation of Neurons Blocks the Inhibition of Neurite Outgrowth by MAG.** Neurite outgrowth was compared, as described in Fig. 1, for cerebellar neurons from PND 2 animals on MAG-expressing and control CHO cells, with and without desialation of the neurons before the assay. C = neurons grown on control CHO cells; MAG = neurons grown on MAG-expressing CHO cells; + = neurons were desialyated prior to the assay; ++ = neurons were desialyated prior to the assay and desialidase was included in the cultures. Results represent the average neurite length ( $\mu$ m) from at least 150 neurons +/- SEM.

**Fig. 6. Desialation of Neurons Blocks the Promotion of Neurite Outgrowth by MAG.** Neurite outgrowth was compared, as described in Fig. 1, for DRG neurons from PND 1 animals on MAG-expressing (MAG) and control CHO cells (Control),  
5 with (MAG sialidase) and without desialylation of the neurons before the assay. Results represent the average neurite length ( $\mu\text{m}$ ) from at least 150 neurons  $\pm$  SEM.

**Fig. 7(a). Soluble MAG-Fc Inhibits Axonal Regeneration of Cerebellar Neurons Grown on L1 in a Concentration-**  
10 **dependent Manner.** L1-Fc was immobilized and isolated PND2 cerebellar neurons were grown overnight in the presence of various concentrations of MAG-Fc (diamonds) or MUC-Fc (squares) as indicated. Neurons were fixed, stained and neurite length measured as described in Fig. 1. Results  
15 represent the average neurite length ( $\mu\text{m}$ )  $\pm$  SEM.

**Fig. 7(b). MAG-Fc Inhibits Axonal Growth in a Specific, Sialic Acid-dependent Manner.** Cerebellar neurons were grown on immobilized L1 as a substrate. MAG-Fc (column 1) or MUC-Fc (column 2) were added at a concentration of 50  
20  $\mu\text{g/ml}$ . Anti-MAG 513 monoclonal antibodies were included at a concentration of 5  $\mu\text{g/ml}$  (column 3) or desialyated neurons were used (column 4). Neurite length ( $\mu\text{m}$ ) was measured as described in Fig. 1. Results represent the average neurite length ( $\mu\text{m}$ )  $\pm$  SEM.

25 **Fig. 7(c). MAG-Fc Inhibits Axonal Growth from Neurons Grown on Fibroblasts.** Isolated PND 2 cerebellar neurons were grown on a substrate of fibroblasts (3T3 cells) in the presence of 50  $\mu\text{g/ml}$  MAG-Fc (Column 1, MAG), 50  $\mu\text{g/ml}$  of MUC18-Fc (column 2, MUC 18) or in the presence of 5  $\mu\text{g/ml}$   
30 anti-MAG 513 monoclonal antibodies (column 3, anti-MAG). Neurite length ( $\mu\text{m}$ ) was measured as described in Fig. 1. Results represent the average neurite length ( $\mu\text{m}$ )  $\pm$  SEM.

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Fig. 8(a). MAG(d1-3)-Fc Binds to Neurons in a Specific, Sialic Acid-dependent Manner. Cerebellar (PND2) neurons vitally labeled with fluorescein were allowed to bind to immobilized MAG(d1-5)-Fc (dark bars), MAG(d1-3)-Fc (hatched bars) or MUC18-Fc (speckled bars), in the presence (+antiMAG) or absence (-Ab) of an anti-MAG monoclonal antibody. Results represent the amount of Fc-chimera bound (ng).

Fig. 8(b). MAG(d1-3)-Fc Does Not Inhibit Axonal Regeneration. Cerebellar neurons were grown on immobilized L1-Fc, in the presence of 50  $\mu\text{g/ml}$  MAG(d1-5)-Fc, Muc18-Fc, or MAG(d1-3)-Fc. Neurite length ( $\mu\text{m}$ ) was measured as described in Fig. 1. Results represent the average neurite length ( $\mu\text{m}$ )  $\pm$  SEM.

Fig. 8(c). MAG(d1-3)-Fc Reverses Inhibition of Axonal Regeneration by Wildtype MAG Expressed by CHO Cells. Cerebellar neurons (PND2) were grown on MAG-expressing (MAG cells) or control  $\odot$  CHO cells, in the presence (+MAG1-3) or absence of MAG(d1-3)-Fc. Neurite length ( $\mu\text{m}$ ) was measured as described in Fig. 1. Results represent the average neurite length ( $\mu\text{m}$ )  $\pm$  SEM.

#### DETAILED DESCRIPTION OF THE INVENTION

In order that the invention herein described may be fully understood, the following detailed description is set forth.

The term "MAG derivative" refers to a molecule comprising at least one MAG extracellular domain, wherein the MAG molecule has been altered (e.g., by recombinant DNA techniques to make chimera with portions of other molecules fused to the MAG molecule, or by chemical or enzymatic modification) or mutated (e.g., internal deletions, insertions, rearrangements and point mutations). MAG derivatives, unless otherwise noted, retain MAG activity.

The terms "MAG bioactivity" and "MAG biological activity" refer to the ability of a molecule, especially an altered or mutant form of MAG, to inhibit or promote neurite outgrowth of a selected neuronal cell type of a particular age, as detected in a neurite outgrowth assay such as those described herein, in qualitatively the same direction as cell-surface or soluble MAG.

The term "MAG binding activity" refers to the ability of a molecule, especially an altered or mutant form of MAG, to compete with cell-surface MAG or soluble MAG for sialic-acid dependent neuron binding in an assay such as those described herein. For example, preferred inhibitors of MAG retain MAG binding activity but have reduced or absent MAG bioactivity.

The term "MAG activity" refers generically to MAG bioactivity and binding activity as described above.

The term "modified derivative of sialic acid" refers to a sialic acid residue that has been modified chemically or enzymatically, especially to add or exchange chemical groups or side chains onto reactive positions of the molecule. Sialic acids are a family of nine-carbon acidic sugars which are derivatives of neuraminic acid and which are often at the termini of cell-surface carbohydrates. "NeuAc" stands for N-acetylneuraminic acid; "GalNAc" stands for N-acetylgalactosamine.

#### **MAG Is a Potent Inhibitor of Neurite Outgrowth**

As described above, MAG had been shown previously to promote neurite extension from particular types of neurons. In addition, MAG was believed to be involved in the initiation of myelin formation. The present invention demonstrates a novel role for MAG as an inhibitor of axonal outgrowth and hence of nerve regeneration in the central nervous system (CNS) and the peripheral nervous system (PNS).

Selected neuronal cell types may be isolated from animals at increasing times in postnatal days (PND)

according to the procedures described in **Example 1**.  
Neurons representing a single cell type may be isolated and  
tested alone, or if desired, mixed populations of cells  
comprising one or more neuronal cell types in the presence  
5 or absence of non-neuronal cells may also be tested.

The present invention provides an in vitro assay  
for determining whether neurite outgrowth from a particular  
type of neuron at a particular age is stimulated or  
inhibited in the presence of MAG. Isolated neurons of  
10 choice may be cultured on a monolayer comprising a growth-  
permissive substrate in the presence or absence of bound  
MAG, and comparative neurite outgrowth may be measured.  
Preferably, the growth-permissive substrate comprises  
mammalian fibroblast cells which have been engineered to  
15 express MAG on their cell surfaces. MAG-expressing cells  
may be engineered using the procedures described in **Example**  
2. Neurite outgrowth on MAG-expressing cells may then be  
compared to neurite outgrowth on control cells that do not  
express cell-surface MAG.

20 As shown in **Fig. 1**, MAG is a potent inhibitor of  
axonal growth from cerebellar neurons from all ages of rats  
tested, newborn to adult. This was determined by  
co-culturing the neurons on transfected Chinese hamster  
ovary (CHO) cells expressing MAG and comparing the length  
25 of the neurites extended to those extended on control  
transfected CHO cells not expressing MAG (see **Example 2**).  
Four different MAG-expressing cell lines were used and each  
had the same effect and inhibited neurite outgrowth by at  
least 70% compared to control cells at all ages tested  
30 (**Fig. 1**; see also Mukhopadhyay et al., *Neuron*, 13, pp. 757-  
67 (1994)). The reversal of neurite growth inhibition by  
anti-MAG antibodies and the lack of effect of CHO cells  
expressing another myelin protein, Po, on neurite outgrowth  
demonstrates that this inhibition is specific to MAG  
35 (Mukhopadhyay et al., *supra*). However, as discussed above,  
it had been reported previously that MAG promotes neurite

outgrowth from newborn DRG neurons (Johnson et al., Neuron, 3, pp. 377-385 (1989)).

To clarify this apparent discrepancy, dorsal root ganglia (DRG) neurons from one- to twenty-day old rats were  
5 tested in the neurite outgrowth assay on CHO cells as described in **Example 2**. As shown in **Fig. 2**, MAG enhanced neurite outgrowth of DRG neurons from two-day old rats; neurites were almost twice as long on MAG-expressing cells compared to control cells. On the other hand, when DRG  
10 neurons from adult rats were tested, MAG inhibited neurite outgrowth by about 40%. A more detailed time course of the effect of MAG on neurite outgrowth from DRG neurons of different ages revealed that the transition from promotion to inhibition takes place at about post-natal day 4 (**Fig.**  
15 **2**). Hence, depending on the age and the type of neuron, MAG can either promote or inhibit neurite outgrowth.

**MAG inhibits axonal outgrowth from many types of neurons**

Characterization of the effect of MAG on a variety of neuronal populations would aid in defining the  
20 requirements for enhanced regeneration after injury in various regions of the nervous system. To establish how other populations of neurons behave in response to MAG, various different neuronal cell types were isolated from one-day old rats (PND1) as described in **Example 1**.  
25 Isolated retinal ganglion (RG), hippocampal (HN), motor neurons (MN) and superior-cervical ganglion (SCG) were tested in CHO cell neurite outgrowth assays as described in **Example 2**. As shown in **Fig. 3**, MAG is a potent inhibitor of neurite growth in all cell types tested.  
30 Thus, MAG likely plays an important role in the lack of neural regeneration in all areas of the nervous system tested to date.

These results show that the transfected mammalian cell assay described in **Example 2** is an effective assay  
35 whereby both the inhibition and promotion of neurite outgrowth by MAG can be monitored and characterized. This



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assay can also be used to screen and identify agents that can block (or enhance) MAG bioactivity, thereby altering its inhibition or stimulation of axonal outgrowth in the nervous system (**Example 3**). Such agents are called herein

5 "MAG-dependent neurite growth altering agents."

**MAG inhibits axonal outgrowth by binding to a sialic acid-bearing glycoprotein on neurons**

MAG binds to all types of neurons tested in a sialic acid-dependent fashion (Kelm et al., Curr. Biol., 4, pp. 965-72

10 (1994)). Fig. 4 shows the results of an aqueous MAG-Fc neuron binding assay which was performed essentially as described in Kelm et al. This experiment confirms that the binding of MAG to isolated PDN1 cerebellar neurons (whose outgrowth is inhibited by MAG) is abolished either by

15 inclusion of anti-MAG monoclonal antibody 513 or by sialidase treatment of neurons before the binding reactions. Sialidase is an enzyme which removes sialic acid from glycoconjugates. Similarly, the binding of MAG to isolated PDN1 DRG neurons (whose outgrowth is promoted

20 by MAG) is inhibited by inclusion of anti-MAG monoclonal antibody 513 and to as lesser extent, by sialidase treatment.

To determine if the sialic acid-dependent binding of MAG to neurons is the event that signals inhibition or

25 promotion of axonal growth and regeneration, neurite outgrowth assays such as those described in **Example 2** were performed after the isolated neurons had been treated with sialidase (**Example 7**). The inhibition of axonal regeneration was reversed by about 50% when PND2 cerebellar

30 neurons were desialyated (Fig. 5). Similarly, when newborn (PND1) DRG neurons were desialyated, promotion of axonal outgrowth by MAG was completely abolished (Fig. 6).

Axonal outgrowth assays such as those described in **Example 2** were also performed in the presence of small,

35 free sialic acid-bearing sugars. These sugars can compete with the sialic acid components of the neuronal surface for

MAG binding and thereby block the inhibition (TABLE 1) or promotion (TABLE 2) of neurite growth by MAG. Inclusion of increasing concentrations of either of the small sialic acid-bearing sugars 2,3-dideoxy sialic acid (DD-NANA) or sialo 2,3- $\alpha$  lactose (SL) reversed the inhibition of axonal growth by MAG by between 40-56% (TABLE 1) and abolished the promotion of neurite outgrowth by MAG completely (TABLE 2).

TABLE 1  
SMALL SIALIC ACID-SUGARS BLOCK  
THE INHIBITION OF AXONAL GROWTH BY MAG

|    | <u>Sugar</u> | <u>Conc.</u><br><u>(mM)</u> | <u>Cell</u> | <u>% Reversal of</u><br><u>Inhibition</u> |
|----|--------------|-----------------------------|-------------|---|
| 15 | DD NANA      | 0                           | control     | 0   |
|    | DD NANA      | 20                          | control     | 0   |
|    | DD NANA      | 0                           | MAG         | 0   |
|    | DD NANA      | 1                           | MAG         | 15%                                       |
|    | DD NANA      | 10                          | MAG         | 45%                                       |
| 20 | DD NANA      | 20                          | MAG         | 64%                                       |
|    | SL           | 0                           | control     | 0   |
|    | SL           | 9                           | control     | 0   |
|    | SL           | 0                           | MAG         | 0   |
|    | SL           | 1                           | MAG         | 11%                                       |
| 25 | SL           | 5                           | MAG         | 42%                                       |
|    | SL           | 9                           | MAG         | 26%                                       |

Neurite outgrowth was compared for cerebellar neurons from PND 2 animals, grown on MAG-expressing and control CHO cells as describe in Example 3.

**TABLE 2**  
**SMALL SIALIC-ACID SUGARS BLOCK THE**  
**PROMOTION OF AXONAL GROWTH BY MAG**

|    | <u>Sugar</u> | <u>Conc.</u><br><u>(mM)</u> | <u>Cell</u> | <u>% Reversal</u><br><u>of Promotion</u> |
|----|--------------|-----------------------------|-------------|--|
| 5  | DD NANA      | 0                           | control     | 0  |
|    | DD NANA      | 20                          | control     | 0  |
|    | DD NANA      | 0                           | MAG         | 0  |
|    | DD NANA      | 1                           | MAG         | 0  |
| 10 | DD NANA      | 10                          | MAG         | 51%                                      |
|    | DD NANA      | 20                          | MAG         | 97%                                      |
|    | SL           | 0                           | control     | 0  |
|    | SL           | 18                          | control     | 0  |
|    | SL           | 0                           | MAG         | 0  |
| 15 | SL           | 1                           | MAG         | 0  |
|    | SL           | 5                           | MAG         | 96%                                      |
|    | SL           | 18                          | MAG         | 98%                                      |

Neurite outgrowth was compared for DRG neurons from PND 2 animals, grown on MAG-expressing and control CHO cells as  
 20 describe in **Example 3**.

The experiments described above demonstrate that MAG biological activity is normally dependent on the ability of MAG to bind to a sialic acid-bearing component on the surface of neurons. It is thus envisioned that a  
 25 variety of agents that can disrupt the ability of MAG to bind to this sialic acid-bearing component will function *in vivo* as MAG inhibitors and will thus be useful for regulating, and especially for promoting neuronal growth in the CNS and PNS.

30 Inhibitors of MAG binding activity include but are not limited to anti-MAG antibodies, free sialic acid-bearing sugars, modified derivatives of sialic acid attached to a sugar, a sialic acid-bearing sugar attached to a protein or lipid carrier molecule, a modified sialic  
 35 acid-bearing sugar attached to a protein or lipid carrier molecule and sialic acid glycopeptides or glycoproteins.

As shown above, inhibitors of MAG binding activity also include enzymes that can alter or remove sialic acid residues, especially those having a Neu5Ac $\alpha$ 2-3Gal $\beta$ 1-3GalNAc (3-O) structure, which mediates MAG binding to neuronal surfaces in the PNS or CNS. Preferred compositions of this embodiment comprise sialidase (a neuraminidase) and sialyl transferases that alter the structure and/or lower the effective concentration of Neu5Ac $\alpha$ 2-3Gal $\beta$ 1-3GalNAc ("3-O") sialylated glycans.

10 **Identifying MAG-dependent Growth Regulating Agents**

Putative new MAG-dependent neurite growth regulating agents may be tested using the procedures described in Example 3. A test agent is identified as a MAG inhibitor when it promotes neurite growth from a cell type inhibited by MAG or inhibits neurite growth from a cell type stimulated by MAG. Similarly, an agent is a MAG agonist when it promotes neurite growth from a cell type stimulated by MAG or inhibits neurite growth from a cell type inhibited by MAG.

20 **Soluble MAG is a potent inhibitor of axonal regeneration**

This invention provides a second method for assaying the effects of MAG on axonal growth and for identifying MAG-dependent neurite growth altering agents. The method involves culturing separate samples of a selected neuronal cell type on a growth-permissive substrate lacking MAG in the presence of either a known concentration of a traceable, soluble form of MAG or of a control protein lacking MAG activity. The neuron cultures are then incubated with a known relative concentration of a test agent for a time sufficient to allow neurite growth, and the amount of neurite growth in the cells cultured in the presence or absence of soluble MAG compared (Example 5). An agent that changes the relative growth of neurites from cells cultured in the presence and absence of soluble

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MAG is identified as a MAG-dependent neurite growth altering agent.

In a preferred embodiment, the growth-permissive substrate lacking MAG comprises a monolayer of mammalian  
5 cells that do not express cell-surface MAG, such as COS or NIH 3T3 cells. A wide variety of mammalian cell lines, such as fibroblast and epithelial cells, may be used and are well known to those of ordinary skill in the art. The present invention is not limited by the cell types which  
10 may be employed to make such growth-permissive monolayers that do not comprise bound MAG.

In another preferred embodiment, the growth-permissive substrate lacking MAG comprises an immobilized monolayer of a purified, growth-promoting factor. It is  
15 well known in the art that neuronal cells may be cultured on growth-promoting monolayers comprising collagen or fibronectin. A preferred neuronal growth-promoting factor according to the present invention which may be immobilized onto a monolayer is the L1 glycoprotein.

20 In preferred embodiments of this method, the soluble form of MAG is a MAG-Fc fusion protein, and the soluble control protein lacking MAG activity is a MUC18-Fc fusion protein (**Example 4**). Preferred traceable fusion proteins are radioactively or fluorescently labeled using  
25 commercially available reagents and methods well known in the art.

**Fig. 7a** shows the results of an assay performed according to the procedures described in **Example 4**. In this assay, neurons were grown on a substrate comprising  
30 the purified growth-promoting molecule termed "L1". MAG in a soluble form, consisting of the extracellular domain of MAG fused to the Fc region of IgG (MAG-Fc) was added to the growing neurons (MAG-Fc; **Example 4**). As the concentration of MAG-Fc was increased, inhibition of neurite outgrowth  
35 increased, while a control chimera, MUC18-Fc, at the same concentration had no effect (**Fig. 7a**). Furthermore, inhibition of axonal regeneration by MAG-Fc could be

reversed by either adding a monoclonal antibody directed against MAG or by desialylating the isolated neurons prior to the assay (Fig. 7b). Soluble MAG-Fc can also inhibit axonal regeneration from cerebellar neurons grown on a monolayer of fibroblasts (Fig. 7c).

Importantly, using this assay, an inhibitor of MAG activity was identified which can bind to neurons without inhibiting axonal regeneration and can reverse the inhibitory effects of wildtype MAG: A truncated form of MAG-Fc, consisting of the first three, rather than the normal five Ig-like extracellular domains of MAG fused to an IgG Fc domain ("MAG(d1-3)-Fc"; Example 4), specifically bound to cerebellar neurons (Fig. 8a). Fig. 8a shows that soluble Fc chimera consisting of all five ("MAG(d1-5)") or the first three ("MAG(d1-3)") Ig-like domains of MAG could bind to cerebellar neurons from PND2 rats in a reaction which was completely inhibited by the presence of anti-MAG monoclonal antibodies. A control Fc chimeric protein (MUC18) did not bind to neurons either in the presence or absence of anti-MAG antibodies.

However, when added to neurons growing on a monolayer substrate of L1, unlike the normal MAG-Fc chimera, MAG(d1-3)-Fc chimera had no effect on axonal regeneration (Fig. 8b). It is thus likely that MAG(d1-3)-Fc can compete with full-length MAG for binding to neurons because MAG(d1-3)-Fc at a concentration of 50  $\mu$ g/ml can reverse by about 40% inhibition of axonal regeneration by full-length MAG expressed by CHO cells (Fig. 8c).

The above experiments demonstrate that altered and/or mutated forms of soluble MAG which harbor one or more mutations in the MAG molecule that reduce or eliminate its ability to inhibit or promote neurite outgrowth but do not significantly diminish the binding of the altered or mutant form of MAG to neuronal surfaces may be useful inhibitors of MAG activity when administered in vivo (Example 8). The most preferred altered/mutant forms of

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MAG of the present invention are soluble molecules comprising a truncated form of MAG-Fc consisting of the first three of the five extracellular Ig-like domains of MAG fused to an immunoglobulin Fc domain ("MAG(d1-3)-Fc").

5 It is envisioned that other more specific mutations (especially point mutations or small internal deletions) may be made to MAG Ig-like domains that will also reduce or eliminate its ability to inhibit or promote neurite outgrowth without significantly diminishing the  
10 binding of the mutant form of MAG to neuronal surfaces. A mutational analysis will likely lead to the identification of a localized "MAG neurite growth signaling site" necessary for activating the downstream cellular signals that are involved in mediating neurite growth regulation.  
15 It is envisioned that certain mutations targeted especially to the fourth and fifth domains which are deleted in the MAG(d1-3)-Fc chimeric protein, and/or to the junction between Ig-like domains three and four in MAG will be useful in this regard.

20

#### **Pharmaceutical Compositions and Treatments Using MAG Derivatives and Inhibitors**

The MAG-dependent neurite growth regulating agents of this invention may be formulated into  
25 pharmaceutical compositions and administered in vivo at an effective dose to treat the particular clinical condition addressed. Administration of one or more of the pharmaceutical compositions according to this invention will be useful for regulating and for promoting neural  
30 growth or regeneration in the nervous system, for treating injuries or damage to nervous tissue or neurons, and for treating neural degeneration associated with traumas to the nervous system, disorders or diseases. Such traumas, diseases or disorders include, but are not limited to:  
35 aneurysms, strokes, Alzheimer's disease, Parkinson's disease, Creutzfeldt-Jacob disease, kuru, Huntington's disease, multiple system atrophy, amyotrophic lateral

sclerosis (Lou Gehrig's disease), and progressive supranuclear palsy.

Determination of a preferred pharmaceutical formulation and a therapeutically efficient dose regiment  
5 for a given application is within the skill of the art taking into consideration, for example, the condition and weight of the patient, the extent of desired treatment and the tolerance of the patient for the treatment.

Administration of the MAG derivatives and  
10 inhibitors of this invention, including isolated and purified forms, their salts or pharmaceutically acceptable derivatives thereof, may be accomplished using any of the conventionally accepted modes of administration of agents which are used to treat neuronal injuries or disorders.

15 Soluble altered and mutated forms of MAG such as those described herein are prepared from the culture media of transfected cells, e.g., COS cells (fibroblasts), transfected with expression plasmids encoding the cDNAs for these forms of MAG (**Example 4**). The soluble MAG molecules,  
20 such as MAG-Fc, are secreted by these cells. It is anticipated that, as has been carried out for hybridoma cells that secrete antibodies (Schnell, L. and Schwab, M. E., Nature, 343, pp. 269-72 (1990); Schnell et al., Nature, 367, pp. 170-73 (1993), COS cells or other transfectants  
25 secreting the soluble MAG-Fc chimera may be implanted into damaged spinal cord. The cells will secrete MAG-inhibiting forms of altered or mutated MAG-Fc, which prevents the endogenous MAG from interacting with the neuronal surface and thus prevents inhibition of axonal growth and  
30 regeneration by endogenous MAG.

About  $2 \times 10^6$  transfected COS cells will secrete about 1 mg of MAG-Fc over a 5-day period. A concentration of 50  $\mu\text{g/ml}$  of mutated MAG-Fc effectively reverses the inhibitory effects of wildtype MAG. Finally, within the  
35 perineurium of an adult rat spinal cord is a volume of about 0.5 ml. Therefore, if  $2 \times 10^6$  mutated or altered MAG-Fc-secreting COS cells are implanted into an injured



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spinal cord, then the concentration of MAG-Fc should be maintained at about 400  $\mu\text{g/ml}$ , i.e., 8-fold more concentrated than the concentration shown herein to be effective in cultured cells. Finally, calculations to correct for the difference between the volume of the perineurium of an adult rat spinal cord compared to the subject being treated can be made by one of skill in the art. Transfected cells, secreting other "reversing" mutated forms of MAG or MAG "blocking" peptides can be administered to the site of neuronal injury or degeneration in a similar manner.

Likewise, other MAG inhibitors and regulators of this invention, e.g., sialidases and sialyltransferases, free, protein- or lipid-attached sialic acid-bearing sugars, glycopeptides or glycoproteins, can also be delivered by spinal implantation (e.g., into the cerebrospinal fluid) of cells engineered to secrete MAG regulating agents according to this invention. Cell secretion rates of the agent are measured in cell culture and then extrapolated based on the

Optionally, transfected cells that secrete MAG regulating agents may be encapsulated into immunoisulatory capsules or chambers and implanted into the brain or spinal cord region using available methods that are known to those of skill in the art. See, e.g., WO 89/04655; WO 92/19195; WO93/00127; EP 127,989; U.S. Patent Nos. US 4,298,002; US 4,670,014; US 5,487,739 and references cited therein, all of which are incorporated herein by reference.

For MAG regulating agents that can not be secreted by transfected cells, a pump and catheter-like device may be implanted at the site of injury to administer the agent on a timely basis and at the desired concentration, which can be selected and empirically modified by one of skill in the art. Such pharmaceutical delivery systems are known to those of skill in the art. See, e.g., U.S. Patent No. 4,578,057 and references cited therein, which are incorporated herein by reference.

The pharmaceutical compositions of this invention may be in a variety of forms, which may be selected according to the preferred modes of administration. These include, for example, solid, semi-solid and liquid dosage forms such as tablets, pills, powders, liquid solutions or suspensions, suppositories, and injectable and infusible solutions. The preferred form depends on the intended mode of administration and therapeutic application. Modes of administration may include oral, parenteral, subcutaneous, intravenous, intralesional or topical administration.

The MAG derivatives and inhibitors of this invention may, for example, be placed into sterile, isotonic formulations with or without cofactors which stimulate uptake or stability. The formulation is preferably liquid, or may be lyophilized powder. For example, the MAG derivatives and inhibitors may be diluted with a formulation buffer comprising 5.0 mg/ml citric acid monohydrate, 2.7 mg/ml trisodium citrate, 41 mg/ml mannitol, 1 mg/ml glycine and 1 mg/ml polysorbate 20. This solution can be lyophilized, stored under refrigeration and reconstituted prior to administration with sterile Water-For-Injection (USP).

The compositions also will preferably include conventional pharmaceutically acceptable carriers well known in the art (see for example Remington's Pharmaceutical Sciences, 16th Edition, 1980, Mac Publishing Company). Such pharmaceutically acceptable carriers may include other medicinal agents, carriers, genetic carriers, adjuvants, excipients, etc., such as human serum albumin or plasma preparations. The compositions are preferably in the form of a unit dose and will usually be administered one or more times a day.

The pharmaceutical compositions of this invention may also be administered using microspheres, liposomes, other microparticulate delivery systems or sustained release formulations placed in, near, or otherwise in communication with affected tissues or the bloodstream.

Suitable examples of sustained release carriers include semipermeable polymer matrices in the form of shaped articles such as suppositories or microcapsules. Implantable or microcapsular sustained release matrices  
5 include polylactides (U.S. Patent No. 3,773,319; EP 58,481), copolymers of L-glutamic acid and gamma ethyl-L-glutamate (Sidman et al., Biopolymers, 22, pp. 547-56 (1985)); poly(2-hydroxyethyl-methacrylate) or ethylene vinyl acetate (Langer et al., J. Biomed. Mater. Res., 15,  
10 pp. 167-277 (1981); Langer, Chem. Tech., 12, pp. 98-105 (1982)).

Liposomes containing MAG derivatives and inhibitors can be prepared by well-known methods (See, e.g. DE 3,218,121; Epstein et al., Proc. Natl. Acad. Sci.  
15 U.S.A., 82, pp. 3688-92 (1985); Hwang et al., Proc. Natl. Acad. Sci. U.S.A., 77, pp. 4030-34 (1980); U.S. Patent Nos. 4,485,045 and 4,544,545). Ordinarily the liposomes are of the small (about 200-800 Angstroms) unilamellar type in which the lipid content is greater than about 30 mol.%  
20 cholesterol. The proportion of cholesterol is selected to control the optimal rate of MAG derivative and inhibitor release.

The MAG derivatives and inhibitors of this invention may also be attached to liposomes, which may  
25 optionally contain other agents to aid in targeting or administration of the compositions to the desired treatment site. Attachment of MAG derivatives and inhibitors to liposomes may be accomplished by any known cross-linking agent such as heterobifunctional cross-linking agents that  
30 have been widely used to couple toxins or chemotherapeutic agents to antibodies for targeted delivery. Conjugation to liposomes can also be accomplished using the carbohydrate-directed cross-linking reagent 4-(4-maleimidophenyl) butyric acid hydrazide (MPBH) (Duzgunes et al., J. Cell.  
35 Biochem. Abst. Suppl. 16E 77 (1992)).

### Utility of MAG Derivatives and Inhibitors

The discovery that MAG is a potent inhibitor of axonal regeneration has potential clinical use in the situations of nervous system injury -- both of the peripheral and central nervous systems -- and in particular for CNS injury. The mammalian central nervous system does not regenerate after injury even though there are many molecules present that promote and encourage a nerve to grow. The result is paralysis or brain damage. It has been shown that there are molecules present in the adult CNS that will actively prevent a nerve from regenerating. It is anticipated that if these inhibitory molecules can be first identified and subsequently blocked, then an environment permissive for regeneration could be engineered.

The first step is to identify what the inhibitory molecules are. MAG is the first such molecule to be identified in myelin. Using the assay systems established herein to monitor the inhibitory effects of MAG, strategies can now be designed with MAG as a target such that its inhibitory function is blocked. Such an agent can then be administered to damaged nerves reversing the inhibitory effects of MAG in vivo and allowing nerve regeneration to proceed.

The assays of the present invention are useful for identifying agents likely to reverse inhibition of nerve regeneration by MAG. Using the assay systems described herein, the inhibitory effects of MAG were shown to be blocked/prevented from functioning by agents such as sialidases or small sialic acid-bearing sugars and by soluble, mutated forms of MAG. These agents, or modified forms of these agents that can either increase or decrease their affinity for MAG or for its receptor, may be administered to damaged nerves, reversing the inhibitory effects of MAG in vivo and allowing regeneration to proceed.

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In addition, the properties of MAG as a negative guidance cue can be used to guide regenerating axons to their correct target and keep them on the correct path. For this purpose, MAG, or different domains of MAG, can be administered to the precise regions of the regenerating nervous tissue to contain growth along exact pathways.

As shown herein, MAG binds to a sialic acid-bearing glycoprotein on neurons to bring about inhibition of nerve growth and regeneration in a wide variety of neuronal cell types. And also as shown herein, when the sialic acid residues of neurons are removed with enzymes termed sialidases, these inhibitory effects of MAG are reversed. Similarly, small sialic acid-bearing sugars and derivatives thereof can bind to MAG, prevent it from interacting with the sialic acid glycoprotein on neurons and prevent its inhibition of axonal regeneration. It is anticipated that in vivo, after injury, application of MAG inhibitors such as sialidases, free small sialic-bearing sugars or modifications of sialic acid attached to other sugars, small sialic acid-bearing sugars covalently attached to protein carrier molecules or lipids or small sialic acid glycoproteins or glycopeptides, either individually or in various combinations, will block the inhibitory effects of MAG and/or other inhibitory molecules that act through a sialic acid-bearing receptor, and encourage axonal regeneration to take place. Similarly, small peptides or peptide fragments of MAG, mutated and altered forms of MAG and antibodies to MAG may block the interaction of endogenous MAG with neurons and allow nerve regeneration.

As shown herein, a mutated, soluble form of MAG can bind to neurons, but itself does not inhibit axonal growth. Importantly, this mutated form of MAG can reverse the inhibitory effects of wildtype MAG. Finally, it is envisioned that MAG, MAG derivatives and MAG inhibitors may be used as a guidance cue in precise regions

of the regenerating nervous system to keep growing axons on the correct path and moving towards the correct target.

All references cited herein are hereby incorporated by reference.

5           The following are examples which illustrate the methods of this invention used to identify the MAG-dependent neurite growth altering agents, compositions of this invention which comprise such agents, and methods comprising the administration of those compositions. These  
10 examples should not be construed as limiting: the examples are included for the purposes of illustration only and the present invention is limited only by the claims.

#### EXAMPLE 1

##### Isolation of Different Neuronal Cell Types

15   Neurons were isolated essentially as described in Doherty et al., Nature, 343, pp. 464-66 (1990); Neuron, 5, pp. 209-19 (1990); and Kleitman et al., Culturing Nerve Cells, pp. 337-78, MIT Press, Cambridge, MA/London, England (G. Banker and K. Goslin, Eds.) (1991). Briefly, for animals up to  
20   nine days of age, the cerebellum, retina, hippocampus, and spinal cord were removed from two animals. Like tissue was combined and placed in 5 ml of 0.025% trypsin in PBS, triturated, and incubated for a further 10 minutes (min.) at 37°C. Trypsinization was stopped by addition of 5 ml  
25   DMEM containing 10% fetal calf serum (FCS) and cells were centrifuged at 800 rpm for 6 min. The cells were resuspended to a single cell suspension in 2 ml of SATO containing 2% FCS. For DRG and SCG neurons, ganglia were removed from two animals and incubated in 5 ml of L15  
30   medium containing 0.025% trypsin and 0.3% collagenase type I (Worthington) for 30 min. at 37°C. The ganglia were triturated with a fire-polished Pasteur pipette. Trypsinization was stopped by adding 5 ml of DMEM containing 10% FCS, centrifuged at 800 rpm for 6 min., and  
35   resuspended in 2 ml of SATO containing 2% FCS. Cells were counted with a Coulter counter.

**EXAMPLE 2****Neurite Outgrowth Assays on Transfected CHO cells**Expression of MAG by Transfected CHO Cells

Chinese hamster ovary (CHO) cells deficient in  
5 the dihydrofolate reductase (*dhfr*) gene (Urlaub and Chasin,  
Proc. Natl. Acad. Sci. USA, 77, pp. 4216-20 (1980)) were  
transfected with a MAG-cDNA expression plasmid with the  
*dhfr* gene and the L-MAG cDNA in either a 5'-3' or, as a  
control, a 3'-5' orientation, cells with multiple copies of  
10 *dhfr* were selected by growing in increasing concentrations  
of methotrexate, and the expression of MAG by individual  
transfected CHO cell lines characterized as described in  
Mukhopadhyay et al., Neuron, 13, pp. 757-67 (1994), which  
is incorporated herein by reference. Transfected cells  
15 were maintained in DMEM supplemented with 10% dialyzed FCS,  
proline (40 mg/liter), thymidine (0.73 mg/liter), and  
glycine (7.5 mg/liter) at 37°C in 5% CO<sub>2</sub>.

The MAG-expressing transfected CHO cell line  
("CHO-MAG2") described as MAG2 in that publication was  
20 deposited on June 27, 1996 with the American Type Culture  
Collection (ATCC) (Rockville, MD) according to the  
provisions of the Budapest Treaty, and was assigned the  
ATCC accession number designated: [            ]. All  
restrictions on the availability to the public of the above  
25 ATCC deposit will be irrevocably removed upon the granting  
of a patent on this application.

Neurite Outgrowth Assays

Confluent monolayers of control and MAG-  
expressing CHO cells were established over a 24-hour (h)  
30 period in individual chambers of an 8-well tissue culture  
slide (Lab-Tek). Co-cultures were established as described  
previously (Doherty et al., Nature, 343, pp. 464-66 (1990);  
Neuron, 5, pp. 209-19 (1990); Mukhopadhyay et al., Neuron,  
13, pp. 757-67 (1994)) by adding approximately 5000

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cerebellar, dorsal root ganglion (DRG) and superior-cervical ganglion (SCG) neurons and 10,000 retinal, hippocampal and spinal cord cells to the CHO monolayers. Culture medium was SATO containing 2% FCS.

- 5 Where indicated, 20 mU of VCS was included throughout the coculture period (see **Example 4**), or monolayers were incubated with small oligosaccharides for one hour before adding the neuronal cell suspension and included throughout the coculture period. After periods of time as indicated,
- 10 the cocultures were fixed for 30 min with 4% paraformaldehyde and permeabilized with ice-cold methanol for 2 min. The cells were then blocked for 30 min with DMEM containing 10% FCS and incubated for 2 h with a rabbit polyclonal antibody against the neuronal marker GAP43
- 15 (1:4000). Cells were washed three times with PBS-BSA (2%) and then incubated for 30 min at room temperature with a biotinylated donkey anti-rabbit Ig (1:300, Amersham), washed three times, and incubated with streptavidin-conjugated Texas Red (1:300, Amersham) for 30 min. After
- 20 three more washes, the slides were mounted in Permfluor (Baxter) and viewed with a Zeiss fluorescent microscope. The length of the longest neurite for each GAP43-positive neuron was determined using the Biological Detection System image analysis program (Pittsburgh).
- 25 Alternatively, other neuron-specific antibodies such as anti-neurofilament monoclonal antibodies, which are commercially available (e.g., Boehringer Mannheim, Sigma Immunochemicals), may be used starting at dilutions recommended by the manufacturer. The appropriate species-
- 30 specific, biotinylated anti-Ig secondary antibody is then selected according to the species in which the primary anti-neural antibody was generated. In addition, various vital dyes (e.g., Molecular Probes, Oregon) which stain neurites may be used in this assay in place of a
- 35 fluorescent neural-specific antibody.



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**EXAMPLE 3****Neurite Growth Assays with CHO Cells to Test Putative MAG-dependent Neurite Growth Altering Agents**

The transfected CHO cell assay described in **Example 2** may  
5 also be used to screen and identify agents that alter  
neurite growth properties of a particular neuronal cell  
type and age in a MAG-dependent fashion. Neurite outgrowth  
was compared for cerebellar (**TABLE 1**) and DRG (**TABLE 2**)  
neurons from PND 2 animals, grown on MAG-expressing and  
10 control CHO cells as described in **Example 2**. Where  
indicated, small sialic acid-bearing sugars were included  
in the co-cultures at increasing concentrations. 100%  
inhibition was taken as the difference in length of  
neurites on control and MAG-expressing CHO cells. Results  
15 are the mean of at least two experiments, with at least 150  
neurons measured for each experiment. DD-NANA =  
2,3-dideoxy sialic acid; SL = sialo 2,3- $\alpha$  lactose.

This assay may be used to test other putative  
MAG-dependent neurite growth regulating agents by including  
20 them in the coculture and measuring their effect in the  
presence and absence of cell-surface MAG as described above  
for the small sialic acid-bearing sugars.

**EXAMPLE 4****Binding of Soluble MAG-Fc Chimeras to Neurons**25 Production of Immunoglobulin Fc-chimeric proteins

Expression plasmids encoding various forms of  
MAG-Fc (such as those referred to herein as MAG[d1-5]-Fc),  
MAG[d1-3]-Fc and a control Fc-chimeric protein MUC 18-Fc)  
were prepared as described in Kelm et al., Current Biol.,

4, pp. 965-72 (1994) and references cited therein. For discussions and a general protocol for making soluble recombinant adhesin molecules, see D.L. Simmons, "Cloning cell surface molecules by transient expression in mammalian cells," in Cellular Interactions in Development - A Practical Approach, pp. 118-125, IRL Press, Oxford (Ed. D.A. Hartley) (1993); Development (Supp.), pp. 193-203 (1993); and P.R. Crocker and S. Kelm, "Methods for studying the cellular binding properties of lectin-like receptors," in Handbook of Experimental Immunology, pp. 1-30 (1995), which are incorporated herein by reference.

*E. coli* cell samples transformed with plasmids that express the MAG[d1-5]-Fc, MAG[d1-3]-Fc and MUC18-Fc chimeric proteins described in Kelm et al., *supra*, were deposited on June 27, 1996 with the American Type Culture Collection (ATCC) (Rockville, MD) according to the provisions of the Budapest Treaty, and were assigned the ATCC accession numbers designated as shown below:

|    | <u>CELL LINE</u> | <u>ATCC<br/>Accession No.</u> |
|----|------------------|-------------------------------|
| a) | MAG[1-3]-Fc      |                               |
| b) | MAG[1-5]-Fc      |                               |
| c) | MUC18-Fc         |                               |

All restrictions on the availability to the public of the above ATCC deposits will be irrevocably removed upon the granting of a patent on this application.

#### Binding of Fc-Chimeras to Neurons

Plasmids encoding MAG-Fc, MAG[d1-3]-Fc and MUC 18-Fc were transfected into COS cells and the Fc-chimeric proteins purified from the media as described in Kelm et al., Current Biol., 4, pp. 965-72 (1994) and P.R. Crocker and S. Kelm, "Methods for studying the cellular binding properties of lectin-like receptors," in Handbook of Experimental Immunology, pp. 1-30 (1995).

Neuron binding assays were performed essentially as

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described in DeBellard et al., Mol. Cell. Neuroscience, 7, pp. 89-101 (1996), which is incorporated herein by reference. Fc-chimeric proteins were adsorbed for 3 h at 37°C to wells of microtiter plates that had been coated for 2 h at 37°C with anti-human IgG at 15 µg/ml in 0.1M bicarbonate buffer, pH 9.6. Prior to the binding assay, neurons were vitally labeled with the fluorescent dye calcein AM (Molecular Probes) by incubating  $2 \times 10^6$  neurons in 5 ml of 10 µM calcein AM in PBS for 15 min at 37°C before being washed and resuspended in PBS. Where indicated, a monoclonal antibody directed against MAG (Boehringer Mannheim) was included in the assay at a concentration of 10 µg/ml, and where indicated, neurons were desialyated before being used. One hundred µl of a suspension of vitally labeled neurons, containing  $1-2 \times 10^5$  cells was added to each well and allowed to incubate for 1 h at room temperature. The plates were washed three times with PBS applied to each well under gravity and the fluorescence was measured in a FluorImager (Molecular Dynamics).

#### EXAMPLE 5

##### Neurite Outgrowth Assays on a Growth Permissive Substrate in the Presence or Absence of MAG-Fc Chimera

##### Growth Permissive Substrate Comprising an L1-Fc Chimera

The L1 glycoprotein is a cell adhesion molecule (CAM) expressed on the surface of a wide variety of mammalian neuronal cell types which stimulates neurite outgrowth. Soluble L1-Fc chimera may be constructed using procedures known to those of skill in the art (such as those cited in Example 3; Doherty et al., Neuron, pp. 57-66 (1995), incorporated herein by reference). Soluble L1-Fc chimera, when presented to neurons, are as effective at promoting neurite outgrowth as the normal cell surface-associated L1 (Doherty et al., supra, and references cited therein which are incorporated herein by reference). As described in Doherty et al., L1-Fc chimera can stably

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associate with the surface of fibroblast 3T3 cells or polylysine/ collagen or polylysine/fibronectin-coated substrates.

Individual wells of an eight-chamber tissue culture plastic slide (Lab-Tek, Nuc. Inc.) were incubated with 0.3 ml of 16.6  $\mu\text{g/ml}$  poly-l-lysine in sterile water for at least one hour under sterile conditions. Each well was washed twice with 400  $\mu\text{l}$  of a 0.1M sodium bicarbonate solution, pH 9.6, and then received 0.3 ml of 0.1M sodium bicarbonate solution, pH 9.6, containing 15  $\mu\text{g/ml}$  goat anti-human IgG (Fc-specific) monoclonal antibody (Sigma). The wells were incubated for 2 h at 37°C, and washed three times with 0.4 ml of ice-cold DMEM. Each well then received 0.3 ml of DMEM containing 40  $\mu\text{g/ml}$  of L1-Fc and was incubated for 2-4 hours at 37°C. The wells were washed twice DMEM.

Neurite Outgrowth on L1-Fc Substrate:

A Soluble MAG-Fc Binding Assay

Cerebellar neurons (post-natal days 2-7) were dissociated by trypsinization as described in Example 1, except that the dissociated neurons were resuspended in 5 ml of SATO medium containing 2% dialyzed FBS. To an individual well coated with a monolayer of L1-Fc as described above,  $5.0 \times 10^4$  cerebellar neurons were added, followed by either a single concentration (about 50  $\mu\text{g/ml}$ ) or increasing concentrations (e.g., 0-30  $\mu\text{g/ml}$ ) of MAG-Fc or MUC18-Fc chimeric soluble proteins, depending on the experiment. Neurons were cultured overnight (about 16 h) at 37°C, and then fixed and stained essentially as described in Example 2.

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**EXAMPLE 6****Neurite Outgrowth Assays Using Soluble MAG To Identify MAG-Dependent Neurite Growth Altering Agents**

The neurite outgrowth binding assay using soluble  
5 MAG-Fc described in **Example 5** may also be used to perform competitive neuron binding/growth experiments to screen and identify new agents that alter the neurite growth properties of a particular neuronal cell type and age in a MAG-dependent fashion. One or more concentrations of the  
10 test agent were included in the cocultures of **Example 5**, and the effect of the test agent in the presence and absence of soluble MAG assessed.

**EXAMPLE 7****Neurite Outgrowth Assays With Desialyated Neurons**

15 Single cell suspensions of different neurons at various postnatal ages were washed and resuspended in phosphate-buffered saline (PBS). Approximately  $2 \times 10^6$  cells were incubated with 50 mU of *Vibrio cholera* sialidase (VCS, Calbiochem) (a neuraminidase) in a final volume of  
20 0.5 ml for 2 hours at 37°C. Neurons were washed with PBS and resuspended in SATO medium containing 2% FCS for neurite outgrowth experiments, or in PBS for neurite binding assays.

This procedure may be modified by using enzymes  
25 other than sialidase that digest or otherwise modify carbohydrate structures (see, e.g., Kelm et al., Carbohydr. Res., 149, pp. 59-64 (1986), which is incorporated by reference herein). For example, sialyl transferases may be employed to alter or remove sialylated glycans on neuronal  
30 surfaces comprising sialic acid residues having a Neu5Ac $\alpha$ 2-3Gal $\beta$ 1-3GalNAc (3-O) structure to which MAG binds.

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**EXAMPLE 8**  
**In Vivo Delivery of MAG-Dependent**  
**Neurite Growth Altering Agents**

5           COS cells transfected with an expression plasmid  
that encodes MAG(d1-3)-Fc were cultured and the cultures  
assayed for the rate of MAG(d1-3)-Fc secretion.  
Approximately  $2 \times 10^6$  cells -- which secrete about 1 mg of  
MAG(d1-3)-Fc over a 5-day period -- are surgically  
10 implanted into the cerebrospinal fluid surrounding the  
spinal cord of an injured subject in the vicinity of nerve  
damage in need of repair. Optionally, repeated  
administrations are performed. The cells secrete MAG(d1-  
3)-Fc, which is capable of inhibiting endogenous MAG  
15 activity in the myelin of the implant site, and neural  
regeneration is stimulated.

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CUNY-9601PCT

International application No.

## INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

|  |  |
|--|--|
| A. The indications made below relate to the microorganism referred to in the description<br>on page <u>29</u> line S <u>17-22</u>  |  |
| B. IDENTIFICATION OF DEPOSIT <span style="float: right;">Further deposits are identified on an additional sheet <input checked="" type="checkbox"/></span>   |  |
| Name of depositary institution<br><u>American Type Culture Collection</u>  |  |
| Address of depositary institution (including postal code and country)<br><u>12301 Parklawn Drive</u><br><u>Rockville, Maryland 20852</u><br><u>United States of America</u>  |  |
| Identification Reference by Depositor: <u>CHO-MAG2</u>   |  |
| Date of deposit<br><u>27 June 1996 (27.06.96)</u>  | Accession Number   |
| C. ADDITIONAL INDICATIONS (leave blank if not applicable) <span style="float: right;">This information is continued on an additional sheet <input checked="" type="checkbox"/></span>  |  |
| In respect of the designation of the EPO, samples of the deposited microorganisms will be made available until the publication of the mention of the grant of the European patent or until the date on which the application is refused or withdrawn or is deemed to be withdrawn, as provided in Rule 28(3) of the Implementing Regulations under the EPC only by the issue of a sample to an expert nominated by requester (Rule 28(3) EPC). |  |
| D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)   |  |
| <u>EPO</u>   |  |
| E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)  |  |
| The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g. "Accession Number of Deposit")  |  |
| <u>Accession Number of Deposit</u>   |  |
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## INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

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| A. The indications made below relate to the microorganism referred to in the description<br>on page <u>29</u> line S <u>17-22</u>   |  |
| B. IDENTIFICATION OF DEPOSIT <span style="float: right;">Further deposits are identified on an additional sheet <input checked="" type="checkbox"/></span>  |  |
| Name of depositary institution<br><u>American Type Culture Collection</u>   |  |
| Address of depositary institution (including postal code and country)<br><u>12301 Parklawn Drive</u><br><u>Rockville, Maryland 20852</u><br><u>United States of America</u>   |  |
| Identification Reference by Depositor: <u>CHO-MAG2</u>  |  |
| Date of deposit<br><u>27 June 1996 (27.06.96)</u>   | Accession Number   |
| C. ADDITIONAL INDICATIONS (leave blank if not applicable) <span style="float: right;">This information is continued on an additional sheet <input checked="" type="checkbox"/></span>   |  |
| <p>In respect of the designation of Finland, until the application has been laid open to public inspection by the Finnish Patent Office, or has been finally decided upon by the Finnish Patent Office without having been laid open to public inspection, samples of the deposited microorganisms will be made available only to an expert in the art.</p> |  |
| D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)  |  |
| <u>Finland</u>  |  |
| E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)   |  |
| The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., 'Accession Number of Deposit')  |  |
| <u>Accession Number of Deposit</u>  |  |
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## INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

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| A. The indications made below relate to the microorganism referred to in the description on page <u>29</u> line S <u>17-22</u>   |  |
| B. IDENTIFICATION OF DEPOSIT   |  |
| Further deposits are identified on an additional sheet <input checked="" type="checkbox"/>   |  |
| Name of depositary institution<br>American Type Culture Collection   |  |
| Address of depositary institution (including postal code and country)<br>12301 Parklawn Drive<br>Rockville, Maryland 20852<br>United States of America   |  |
| Identification Reference by Depositor: CHO-MAG2  |  |
| Date of deposit<br>27 June 1996 (27.06.96)   | Accession Number   |
| C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is continued on an additional sheet <input type="checkbox"/>  |  |
| Applicant(s) hereby give notice of my/our intention that samples of the above-identified culture shall be available only to experts in accordance with paragraph 3 of the Fourth Schedule to the Patents Rules 1995. |  |
| D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)   |  |
| Singapore  |  |
| E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)  |  |
| The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., 'Accession Number of Deposit')   |  |
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## INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description  
on page 32 line 21

## B. IDENTIFICATION OF DEPOSIT

Further deposits are identified on an additional sheet ☒

Name of depositary institution

American Type Culture Collection

Address of depositary institution (including postal code and country)

12301 Parklawn Drive  
Rockville, Maryland 20852  
United States of America

Identification Reference by Depositor: MAG (1-3)-Fc

Date of deposit

27 June 1996 (27.06.96)

Accession Number

## C. ADDITIONAL INDICATIONS (leave blank if not applicable)

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In respect of the designation of the EPO, samples of the deposited microorganisms will be made available until the publication of the mention of the grant of the European patent or until the date on which the application is refused or withdrawn or is deemed to be withdrawn, as provided in Rule 28(3) of the Implementing Regulations under the EPC only by the issue of a sample to an expert nominated by requester (Rule 28(4) EPC).

## D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)

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## E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)

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## INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

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| A. The indications made below relate to the microorganism referred to in the description<br>on page <u>32</u> line <u>21</u>   |  |
| B. IDENTIFICATION OF DEPOSIT   |  |
| Further deposits are identified on an additional sheet <input checked="" type="checkbox"/>   |  |
| Name of depositary institution<br>American Type Culture Collection   |  |
| Address of depositary institution (including postal code and country)<br>12301 Parklawn Drive<br>Rockville, Maryland 20852<br>United States of America   |  |
| Identification Reference by Depositor: MAG (1-3)-Fc  |  |
| Date of deposit<br>27 June 1996 (27.06.96)   | Accession Number   |
| C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is continued on an additional sheet <input checked="" type="checkbox"/>   |  |
| In respect of the designation of Finland, until the application has been laid open to public inspection by the Finnish Patent Office, or has been finally decided upon by the Finnish Patent Office without having been laid open to public inspection, samples of the deposited microorganisms will be made available only to an expert in the art. |  |
| D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)   |  |
| Finland  |  |
| E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)  |  |
| The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")   |  |
| Accession Number of Deposit  |  |
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## INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

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| A. The indications made below relate to the microorganism referred to in the description on page <u>32</u> line <u>21</u>  |  |
| B. IDENTIFICATION OF DEPOSIT <span style="float: right;">Further deposits are identified on an additional sheet <input checked="" type="checkbox"/></span>   |  |
| Name of depositary institution<br>American Type Culture Collection   |  |
| Address of depositary institution (including postal code and country)<br>12301 Parklawn Drive<br>Rockville, Maryland 20852<br>United States of America   |  |
| Identification Reference by Depositor: MAG (1-3)-Fc  |  |
| Date of deposit<br>27 June 1996 (27.06.96)   | Accession Number   |
| C. ADDITIONAL INDICATIONS (leave blank if not applicable) <span style="float: right;">This information is continued on an additional sheet <input type="checkbox"/></span>   |  |
| Applicant(s) hereby give notice of my/our intention that samples of the above-identified culture shall be available only to experts in accordance with paragraph 3 of the Fourth Schedule to the Patents Rules 1995. |  |
| D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)   |  |
| Singapore  |  |
| E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)  |  |
| The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")<br><br>Accession Number of Deposit              |  |
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| A. The indications made below relate to the microorganism referred to in the description<br>on page <u>32</u> line <u>22</u>   |  |
| B. IDENTIFICATION OF DEPOSIT   |  |
| Further deposits are identified on an additional sheet <input checked="" type="checkbox"/>   |  |
| Name of depositary institution<br>American Type Culture Collection   |  |
| Address of depositary institution (including postal code and country)<br>12301 Parklawn Drive<br>Rockville, Maryland 20852<br>United States of America   |  |
| Identification Reference by Depositor: MAG (1-5)-Fc  |  |
| Date of deposit<br>27 June 1996 (27.06.96)   | Accession Number   |
| C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is continued on an additional sheet <input checked="" type="checkbox"/>   |  |
| In respect of the designation of the EPO, samples of the deposited microorganisms will be made available until the publication of the mention of the grant of the European patent or until the date on which the application is refused or withdrawn or is deemed to be withdrawn, as provided in Rule 28(3) of the Implementing Regulations under the EPC only by the issue of a sample to an expert nominated by requester (Rule 28(4) EPC). |  |
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| EPO  |  |
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| B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet <input checked="" type="checkbox"/>  |  |
| Name of depositary institution<br>American Type Culture Collection   |  |
| Address of depositary institution (including postal code and country)<br>12301 Parklawn Drive<br>Rockville, Maryland 20852<br>United States of America   |  |
| Identification Reference by Depositor: MAG (1-5)-Fc  |  |
| Date of deposit<br>27 June 1996 (27.06.96)   | Accession Number   |
| C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is continued on an additional sheet <input checked="" type="checkbox"/>   |  |
| In respect of the designation of Finland, until the application has been laid open to public inspection by the Finnish Patent Office, or has been finally decided upon by the Finnish Patent Office without having been laid open to public inspection, samples of the deposited microorganisms will be made available only to an expert in the art. |  |
| D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)   |  |
| Finland  |  |
| E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)  |  |
| The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")   |  |
| Accession Number of Deposit  |  |
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| Applicant's or agent's file<br>reference number | CUNY-9601PCT | International application No. |
|---|--------------|-------------------------------|

## INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

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| A. The indications made below relate to the microorganism referred to in the description<br>on page <u>32</u> line <u>22</u>   |  |
| B. IDENTIFICATION OF DEPOSIT   |  |
| Further deposits are identified on an additional sheet <input checked="" type="checkbox"/>   |  |
| Name of depositary institution<br>American Type Culture Collection   |  |
| Address of depositary institution (including postal code and country)<br>12301 Parklawn Drive<br>Rockville, Maryland 20852<br>United States of America   |  |
| Identification Reference by Depositor: MAG (1-5)-Fc  |  |
| Date of deposit<br>27 June 1996 (27.06.96)   | Accession Number   |
| C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is continued on an additional sheet <input type="checkbox"/>  |  |
| Applicant(s) hereby give notice of my/our intention that samples of the above-identified culture shall be available only to experts in accordance with paragraph 3 of the Fourth Schedule to the Patents Rules 1995. |  |
| D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)<br>Singapore  |  |
| E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)  |  |
| The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")<br>Accession Number of Deposit                  |  |
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| A. The indications made below relate to the microorganism referred to in the description<br>on page <u>32</u> line <u>23</u>   |  |
| B. IDENTIFICATION OF DEPOSIT <span style="float: right;">Further deposits are identified on an additional sheet <input type="checkbox"/></span>  |  |
| Name of depositary institution<br><u>American Type Culture Collection</u>  |  |
| Address of depositary institution (including postal code and country)<br><u>12301 Parklawn Drive</u><br><u>Rockville, Maryland 20852</u><br><u>United States of America</u>  |  |
| Identification Reference by Depositor: <u>MUC18-Fc</u>   |  |
| Date of deposit<br><u>27 June 1996 (27.06.96)</u>  | Accession Number   |
| C. ADDITIONAL INDICATIONS (leave blank if not applicable) <span style="float: right;">This information is continued on an additional sheet <input checked="" type="checkbox"/></span>  |  |
| In respect of the designation of the EPO, samples of the deposited microorganisms will be made available until the publication of the mention of the grant of the European patent or until the date on which the application is refused or withdrawn or is deemed to be withdrawn, as provided in Rule 28(3) of the Implementing Regulations under the EPC only by the issue of a sample to an expert nominated by requester (Rule 28(4) EPC). |  |
| D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)   |  |
| <u>EPO</u>   |  |
| E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)  |  |
| The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")   |  |
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## INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

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| A. The indications made below relate to the microorganism referred to in the description on page <u>32</u> , line <u>23</u>  |  |
| B. IDENTIFICATION OF DEPOSIT   |  |
| Further deposits are identified on an additional sheet <input type="checkbox"/>  |  |
| Name of depositary institution<br>American Type Culture Collection   |  |
| Address of depositary institution (including postal code and country)<br>12301 Parklawn Drive<br>Rockville, Maryland 20852<br>United States of America   |  |
| Identification Reference by Depositor: MUC18-Fc  |  |
| Date of deposit<br>27 June 1996 (27.06.96)   | Accession Number   |
| C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is continued on an additional sheet <input checked="" type="checkbox"/>   |  |
| In respect of the designation of Finland, until the application has been laid open to public inspection by the Finnish Patent Office, or has been finally decided upon by the Finnish Patent Office without having been laid open to public inspection, samples of the deposited microorganisms will be made available only to an expert in the art. |  |
| D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)<br>Finland  |  |
| E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)  |  |
| The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")<br>Accession Number of Deposit  |  |
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reference number

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International application No.

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(PCT Rule 13bis)

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| B. IDENTIFICATION OF DEPOSIT   |  |
| Further deposits are identified on an additional sheet <input type="checkbox"/>  |  |
| Name of depositary institution<br>American Type Culture Collection   |  |
| Address of depositary institution (including postal code and country)<br>12301 Parklawn Drive<br>Rockville, Maryland 20852<br>United States of America   |  |
| Identification Reference by Depositor: MUC18-Fc  |  |
| Date of deposit<br>27 June 1996 (27.06.96)   | Accession Number   |
| C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is continued on an additional sheet <input type="checkbox"/>  |  |
| Applicant(s) hereby give notice of my/our intention that samples of the above-identified culture shall be available only to experts in accordance with paragraph 3 of the Fourth Schedule to the Patents Rules 1995. |  |
| D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)   |  |
| Singapore  |  |
| E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)  |  |
| The indications listed below will be submitted to the International Bureau later (specify the general nature of the indication e.g., "Accession Number of Deposit")  |  |
| Accession Number of Deposit  |  |
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What is claimed is:

1. A pharmaceutical composition which comprises a pharmaceutically acceptable carrier and a therapeutically effective amount of at least one MAG inhibitor.
- 5 2. The pharmaceutical composition according to claim 1, wherein the MAG inhibitor is selected from the group consisting of an altered or a mutated form of MAG characterized by an altered biological activity, a free sialic acid-bearing sugar, a modified derivative of sialic  
10 acid attached to a sugar, a sialic acid-bearing sugar attached to a protein or lipid carrier molecule, a modified sialic acid-bearing sugar attached to a protein or lipid carrier molecule, and a sialic acid glycopeptide.
3. The pharmaceutical composition according to claim 1,  
15 wherein the MAG inhibitor comprises a sialic acid-bearing sugar.
4. The pharmaceutical composition according to claim 3, wherein the sialic acid is sialo 2,3- $\alpha$  lactose or 2,3-dideoxy sialic acid.
- 20 5. The pharmaceutical composition according to claim 1, wherein the MAG inhibitor comprises an altered form of MAG which can inhibit the binding of endogenous MAG to neurons in the CNS or PNS.
6. The pharmaceutical composition according to claim 5,  
25 wherein the altered form of MAG is a soluble chimeric protein that harbors one or more mutations in the MAG molecule that reduce or eliminate its ability to inhibit or promote neurite outgrowth compared to endogenous or soluble MAG, but do not significantly diminish the binding of the  
30 altered form of MAG to neuronal surfaces.

7. The pharmaceutical composition according to claim 5, wherein the altered form of MAG is a soluble chimeric protein which comprises extracellular Ig-like domains one to three of MAG.
- 5 8. The pharmaceutical composition according to claim 7, wherein the altered form of MAG is MAG(d1-3)-Fc.
9. The pharmaceutical composition according to claim 5, wherein the altered form of MAG is a soluble chimeric  
10 protein which comprises extracellular Ig-like domains one to five of MAG.
10. The pharmaceutical composition according to claim 9, wherein the altered form of MAG is MAG(d1-5)-Fc.
11. The pharmaceutical composition according to claim 1,  
15 wherein the MAG inhibitor is an enzyme that can alter or remove sialyated glycans on neuronal surfaces comprising sialic acid residues having a Neu5Ac $\alpha$ 2-3Gal $\beta$ 1-3GalNAc (3-O) structure.
12. The pharmaceutical composition according to claim 11,  
20 wherein the enzyme is a sialidase.
13. A method for regulating neural growth or regeneration in the nervous system which comprises the step of administering a pharmaceutical composition according to any one of claims 1-12.
- 25 14. A method for regulating neural growth or regeneration in the nervous system which comprises the step of administering a pharmaceutical composition according to any one of claims 1-12.
15. The method according to claim 14, wherein neural  
30 growth or regeneration is promoted.

16. A method for treating injuries or damage to nervous tissue or neurons which comprises the step of administering a pharmaceutical composition according to any one of claims 1-12.

5 17. A method for treating neural degeneration associated with a disorder or disease which comprises the step of administering a pharmaceutical composition according to any one of claims 1-12.

10 18. A method for determining whether neurite outgrowth from a particular type of neuron at a particular age is stimulated or inhibited in the presence of MAG or a MAG derivative which comprises the steps of:

15 a) culturing a first sample of a selected neuronal cell type on a growth-permissive substrate in the absence of MAG;  
b) culturing a second sample of the selected neuronal cell type on a growth-permissive substrate comprising bound MAG; and

20 c) comparing the relative amount of neurite growth in the cultured cells of a) and b);

wherein when the relative growth of neurites in the cultured cells of a) is greater than in b), the neuronal cell is inhibited by the presence of MAG, and when the  
25 relative growth of neurites in the cultured cells of a) is less than in b), the neuronal cell is stimulated by the presence of MAG.

19. The method according to claim 18, wherein the growth-permissive substrate in the absence of MAG comprises a  
30 monolayer of mammalian cells that do not express cell-surface MAG, and the growth-permissive substrate comprising bound MAG comprises a monolayer of equivalent mammalian cells engineered to express cell surface MAG.

20. The method according to claim 19, wherein the mammalian cells are CHO cells engineered to express cell surface MAG.

21. The method according to claim 20, wherein the  
5 mammalian cells are CHO-MAG2 cells.

22. A method for identifying a MAG-dependent neurite growth altering agent for a selected neuronal cell type which comprises the steps of:

a) culturing a first sample of a selected neuronal  
10 cell type on a growth-permissive substrate in the absence of MAG;

b) culturing a second sample of the selected neuronal cell type on a growth-permissive substrate comprising bound MAG in an amount sufficient to alter neurite outgrowth from  
15 the cells compared to the first sample of cells cultured in the absence of MAG;

c) incubating the cell cultures of a) and b) with a known relative concentration of a test agent for a time sufficient to allow neurite growth; and

20 d) comparing the relative amount of neurite growth in the cultured cells of a) and b); wherein an agent that changes the relative growth of neurites in the cultured cells of a) and b) is identified as a MAG-dependent neurite growth altering agent.

25 23. The method according to claim 22, wherein the growth-permissive substrate in the absence of MAG comprises a monolayer of mammalian cells that do not express cell-surface MAG, and the growth-permissive substrate comprising bound MAG comprises a monolayer of equivalent mammalian  
30 cells engineered to express cell surface MAG.

24. The method according to claim 23, wherein the mammalian cells are CHO cells engineered to express cell surface MAG.

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25. The method according to claim 24, wherein the mammalian cells are CHO-MAG2 cells.

26. A method for identifying a MAG-dependent neurite growth altering agent for a selected neuronal cell type  
5 comprises the steps of:

a) culturing separate samples of a selected neuronal cell type on a growth-permissive substrate lacking MAG;

b) culturing a first sample of a) with a known concentration of a traceable, soluble form of MAG;

10 c) culturing a second sample of a) with a known concentration of a traceable, soluble form of a control protein lacking MAG activity;

d) incubating the cultures of b) and c) with a known relative concentration of a test agent for a time  
15 sufficient to allow neurite growth; and

e) comparing the relative amount of neurite growth in the cultured cells of c) and d);

wherein an agent that changes the relative growth of neurites in the cultured cells of c) and d) is identified  
20 as a MAG-dependent neurite growth altering agent.

27. The method according to claim 26, wherein the growth-permissive substrate lacking MAG comprises a monolayer of mammalian cells that do not express cell-surface MAG.

28. The method according to claim 26, wherein the growth-  
25 permissive substrate lacking MAG comprises an immobilized monolayer of a purified, growth-promoting factor.

29. The method according to claim 28, wherein the growth-promoting factor comprises an L1 glycoprotein.

30. The method according to claim 26, wherein the soluble  
30 form of MAG is a MAG-Fc chimeric protein, and the soluble control protein lacking MAG activity is a MUC18-Fc chimeric protein.

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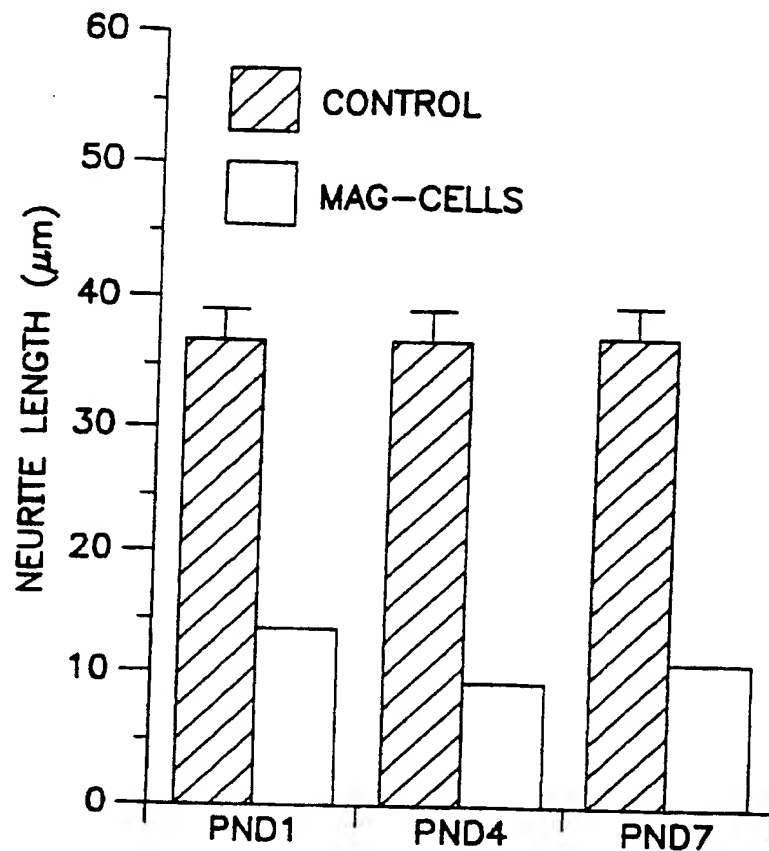


FIG. 1

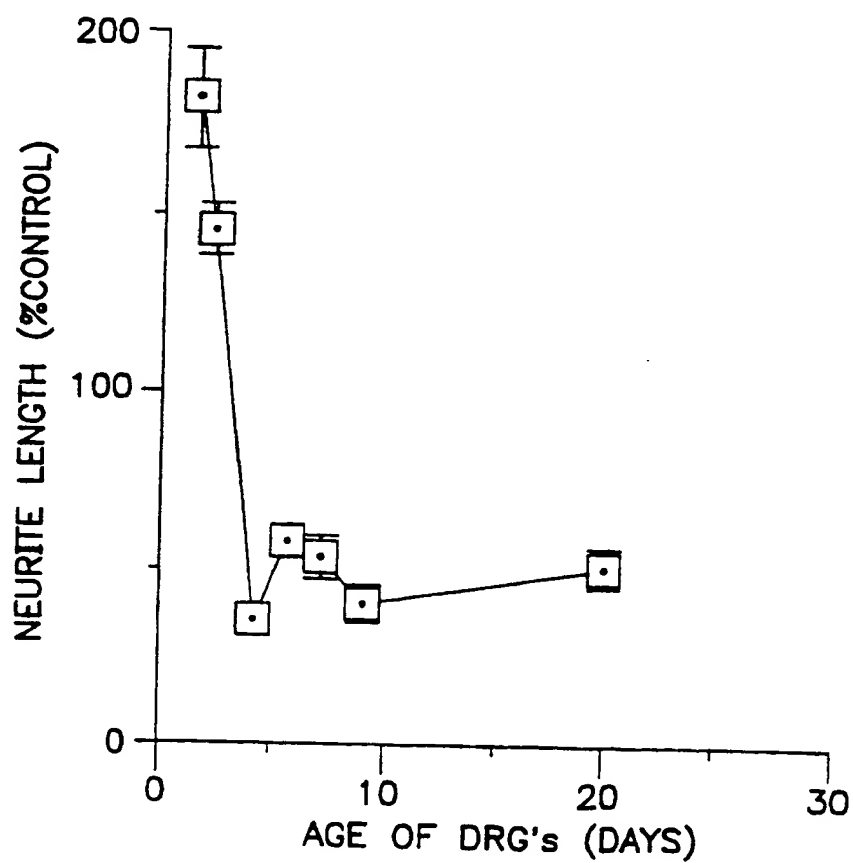


FIG. 2



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FIG. 3

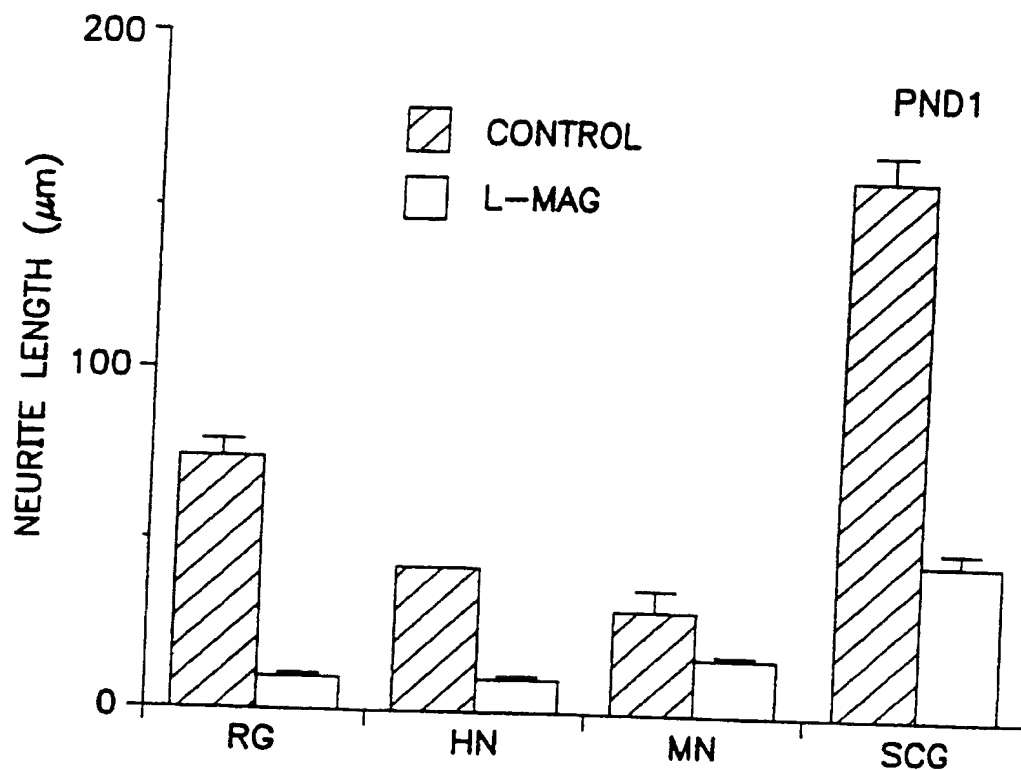
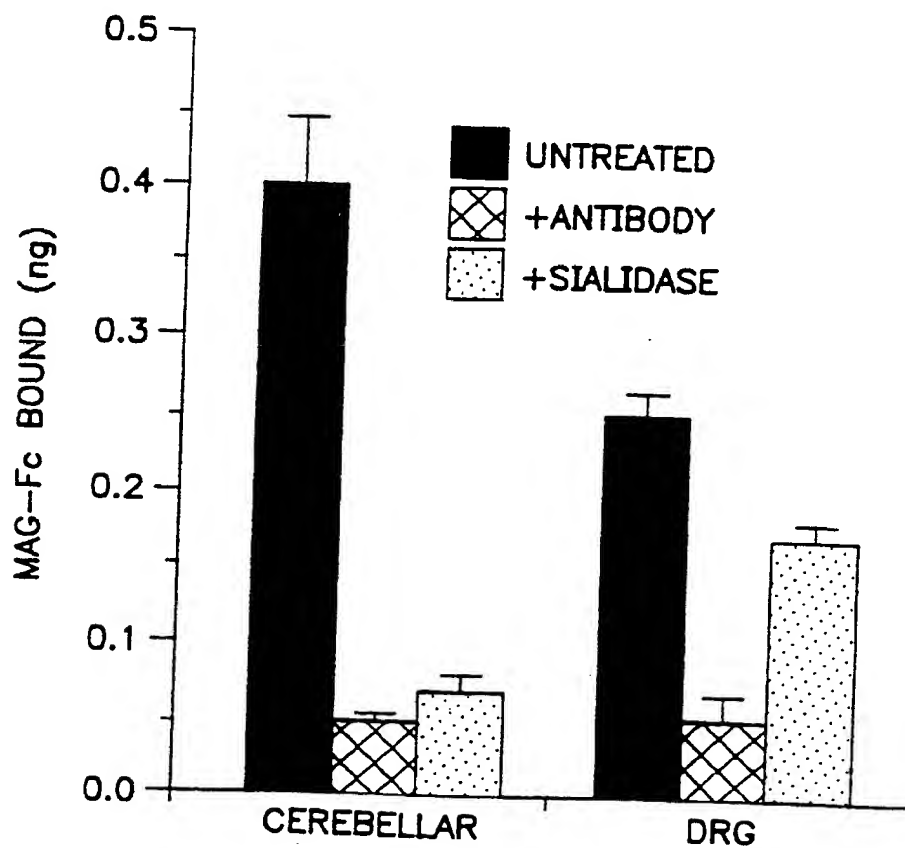


FIG. 4



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FIG. 5

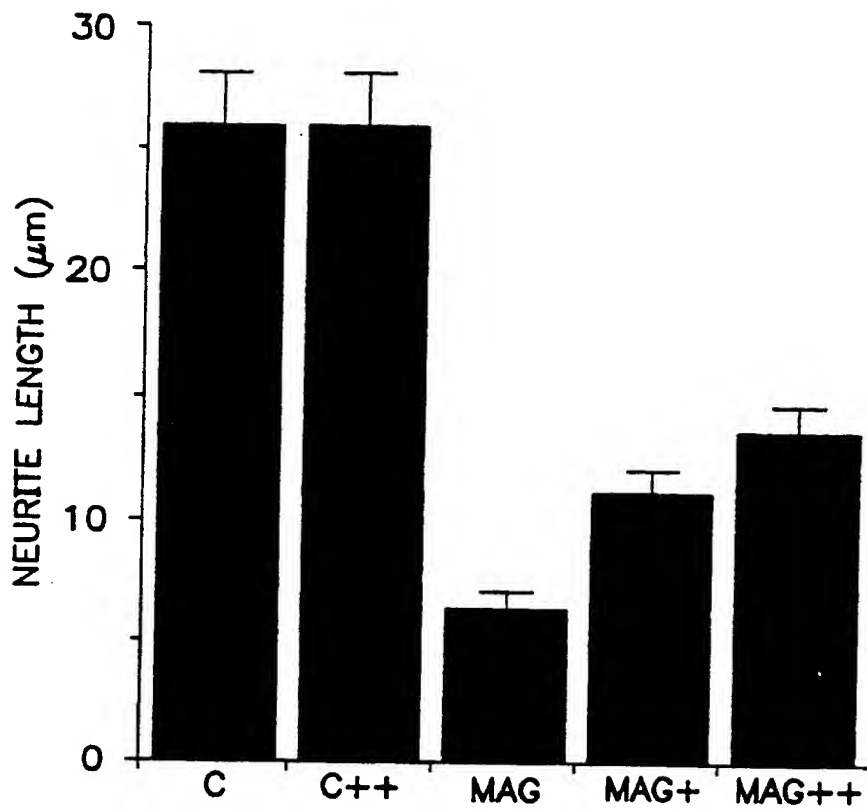
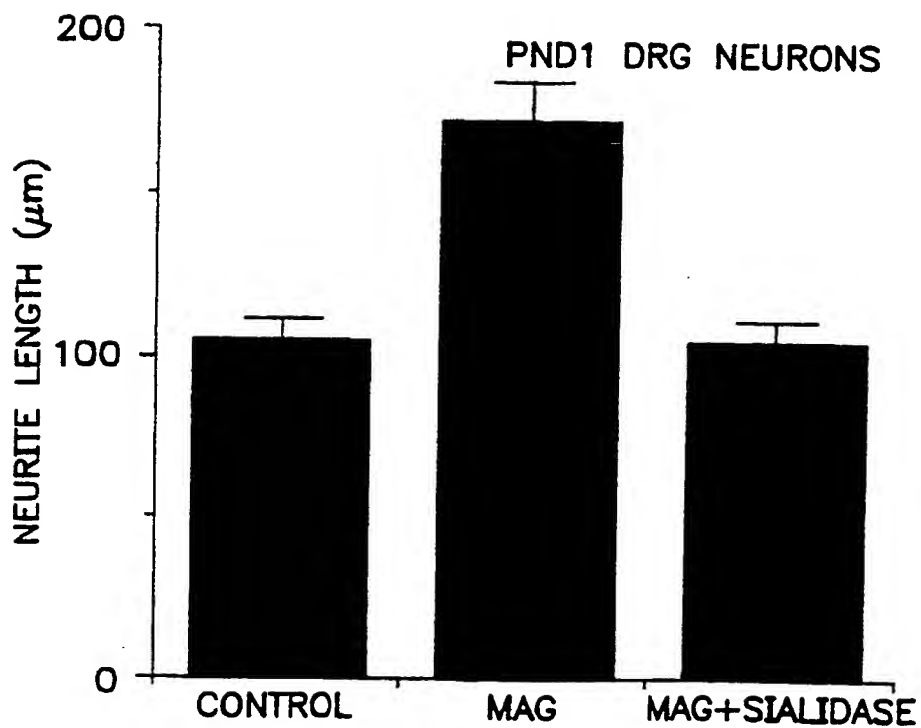


FIG. 6



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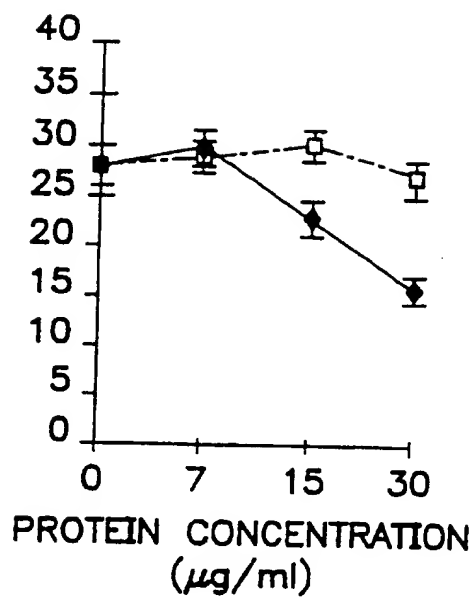


FIG. 7(a)

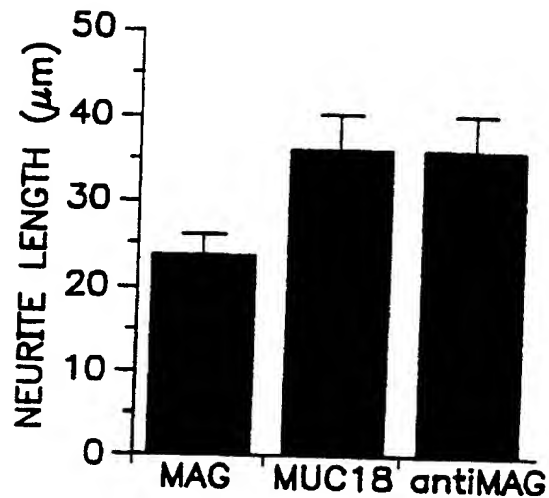


FIG. 7(c)

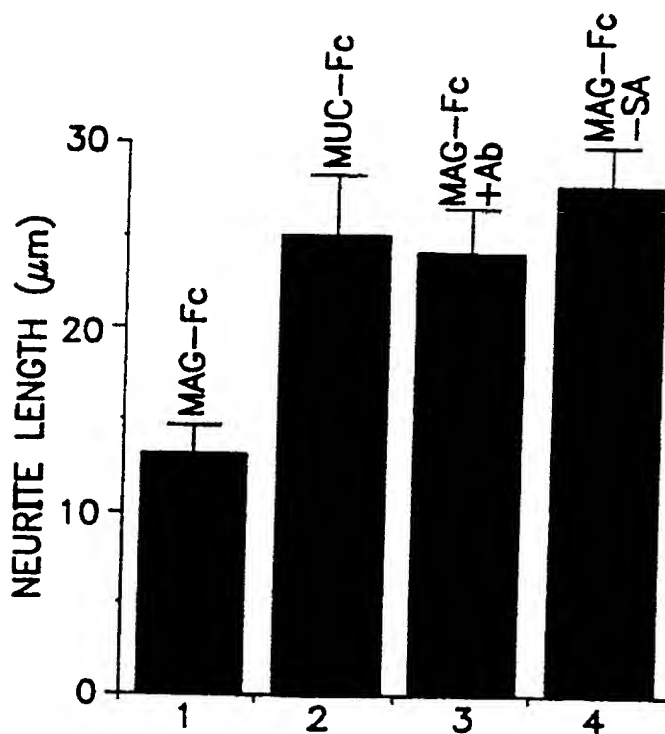


FIG. 7(b)

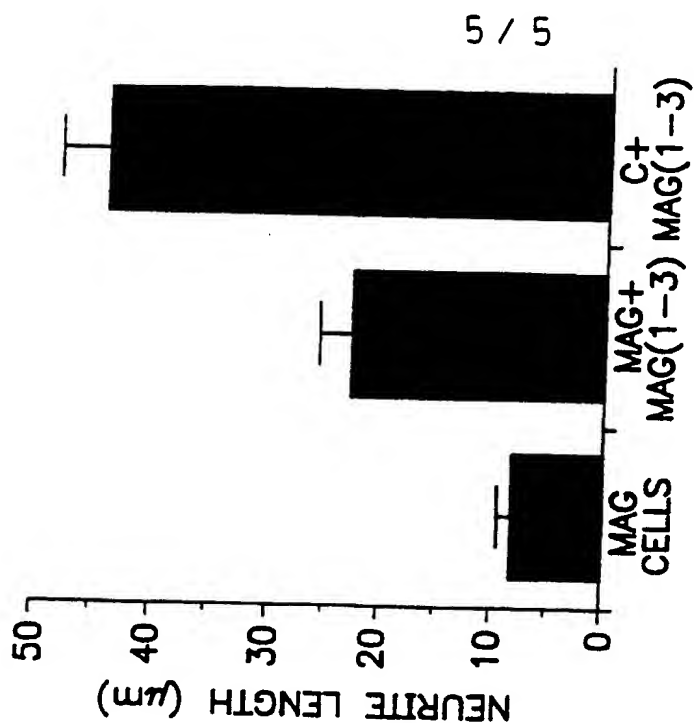


FIG. 8(c)

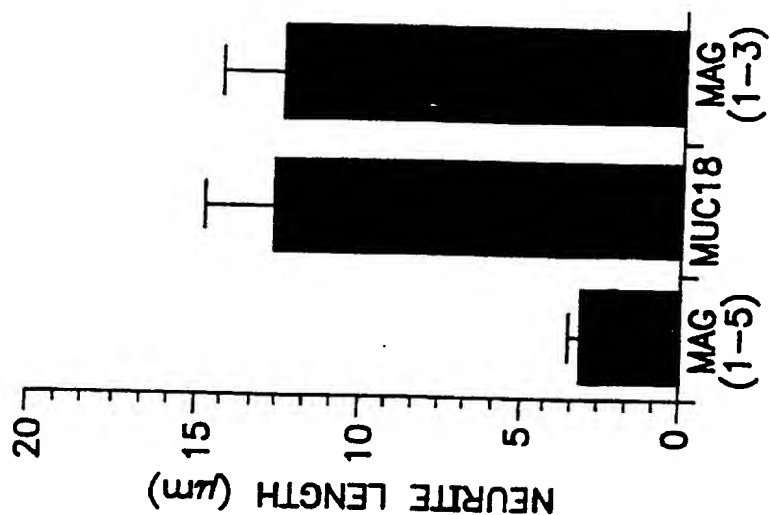
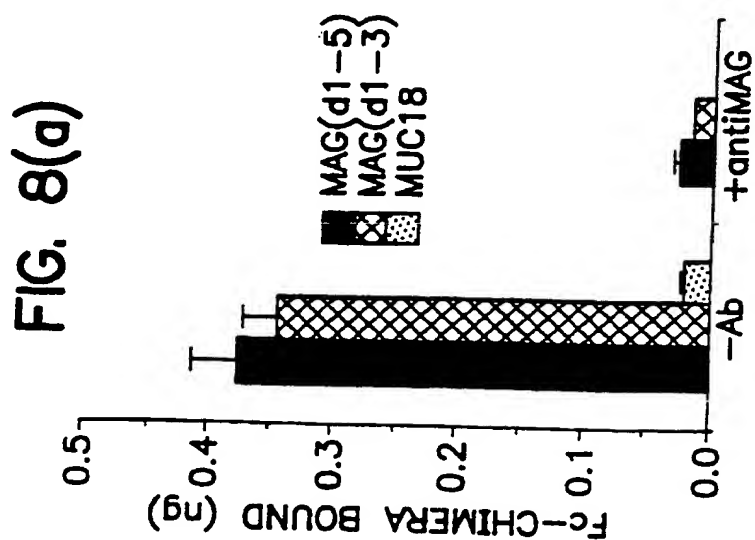


FIG. 8(b)



## INTERNATIONAL SEARCH REPORT

International Application No

PC1/US 96/11058

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 A61K38/17 A61K31/70 G01N33/68 //C07K14/705,C12N15/85

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 A61K C07K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages   | Relevant to claim No.  |
|------------|--|------------------------|
| X          | TWENTY-SIXTH MEETING OF THE AMERICAN SOCIETY FOR NEUROCHEMISTRY, SANTA MONICA, CALIFORNIA, USA, MARCH 5-9, 1995. JOURNAL OF NEUROCHEMISTRY 64 (SUPPL. 1). 1995. S41, XP002016510<br>FILBIN M T ET AL: "MAG as an inhibitor of neurite outgrowth."<br>see the whole document<br>--- | 1,2,<br>5-10,<br>13-30 |
| X          | NEURON (1994), 13(4), 805-11,<br>1994, XP000605260<br>MCKERRACHER, L. ET AL: "Identification of myelin - associated glycoprotein as a major myelin-derived inhibitor of neurite growth"<br>cited in the application<br>see the whole document<br>---<br>-/-                        | 1,2,<br>5-10,<br>13-30 |

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

## \* Special categories of cited documents:

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the international filing date
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- \*O\* document referring to an oral disclosure, use, exhibition or other means
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- \*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- \*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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- \* & \* document member of the same patent family

Date of the actual completion of the international search

23 October 1996

Date of mailing of the international search report

05. 11. 96

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,  
Fax: (+31-70) 340-3016

Authorized officer

Moreau, J

## INTERNATIONAL SEARCH REPORT

Inter- national Application No

PCI/US 96/11058

C(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category | Citation of document, with indication, where appropriate, of the relevant passages   | Relevant to claim No.  |
|----------|--|------------------------|
| X        | NEURON (1994), 13(3), 757-67,<br>1994, XP000605261<br>MUKHOPADHYAY, GITALI ET AL: "A novel role<br>for myelin - associated glycoprotein as an<br>inhibitor of axonal regeneration"<br>cited in the application<br>see the whole document   | 1,2,<br>5-10,<br>13-30 |
| X        | NEURON (1995), 14(1), 57-66 ,<br>1995, XP000605271<br>DOHERTY, PATRICK ET AL: "A soluble<br>chimeric form of the L1 glycoprotein<br>stimulates neurite outgrowth"<br>cited in the application<br>see the whole document  | 1,2,<br>5-10,<br>13-30 |
| P,X      | WO 95 22344 A (MCGILL UNIVERSITY) 24<br>August 1995<br><br>see the whole document  | 1,2,<br>5-10,<br>13-30 |
| P,X      | INTERNATIONAL SYMPOSIA ON MYELIN AND<br>MYELIN-FORMING CELLS, OKAZAKI, JAPAN, JULY<br>8-10, 1995. DEVELOPMENTAL NEUROSCIENCE 17<br>(3). 1995. 183, XP000605479<br>FILBIN M T ET AL: "The effect of myelin -<br>associated glycoprotein on neurite<br>outgrowth is dependent on the interaction<br>with a neuronal sialo-glycoprotein."<br>see the whole document | 1-30                   |
| P,X      | MOLECULAR AND CELLULAR NEUROSCIENCE 7 (2).<br>1996. 89-101, XP000608101<br>DEBELLARD M-E ET AL: "Myelin - associated<br>glycoprotein inhibits axonal regeneration<br>from a variety of neurons via interaction<br>with a sialoglycoprotein."<br>see the whole document   | 1-30                   |
| P,X      | CURRENT OPINION IN NEUROBIOLOGY 5 (5).<br>1995. 588-595, XP000608264<br>FILBIN M T: "Myelin - associated<br>glycoprotein: A role in myelination and in<br>the inhibition of axonal regeneration ?."<br>see the whole document  | 1-30                   |
| P,X      | NEUROSCIENCE LETTERS 205 (2). 1996. 87-90,<br>XP000605913<br>MATSUDA Y ET AL: "Soluble myelin -<br>associated glycoprotein- immunoglobulin G1<br>chimera protein promotes neurite outgrowth<br>from mouse cerebellar neurons."<br>see the whole document   | 1,2,<br>5-10,<br>13-30 |

# INTERNATIONAL SEARCH REPORT

I national application No.

PCT/US 96/11058

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claim(s) 13 - 17  
is(are) directed to a method of treatment of the human/animal  
body, the search has been carried out and based on the alleged  
effects of the compound/composition.

2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such  
an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all  
searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment  
of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report  
covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is  
restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

### Information on patent family members

PC 1/US 96/11058

Form PCT/ISA/210 (patent family annex) (July 1992)